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**(54) Trehalose releasing enzyme, DNA encoding therefor, their preparation and uses.**

**(57) Disclosed are a DNA encoding an enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, recombinant DNA and enzyme, transformant, and their preparations and uses. These facilitate the industrial-scale production of trehalose with a relative easiness and low cost, and trehalose thus obtained can be satisfactorily used in a variety of food products, cosmetics and pharmaceuticals.**

The present invention relates to a novel DNA encoding an enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, recombinant DNA containing the same, and a transformant, and further relates to a recombinant enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, as well as to preparations and uses thereof.

Trehalose is a disaccharide which consists of 2 glucose molecules which are linked together with their reducing groups, and, naturally, it is present in bacteria, fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, trehalose does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can sweeten food products without fear of causing unsatisfactory coloration and deterioration. Trehalose, however, is far from being readily prepared in a desired amount by conventional methods, and, actually, it has not scarcely been used for sweetening food products.

Conventional methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other employing a multi-enzymatic system wherein enzymes are allowed to act on saccharides. The former, as disclosed in Japanese Patent Laid-Open No. 154,485/75, is a method which comprises allowing to grow microorganisms such as bacteria and yeasts in a nutrient culture medium, and collecting trehalose from the proliferated cells in the resultant culture. The latter, as disclosed in Japanese Patent Laid-Open No. 216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and trehalose-phosphorylases to act on maltose, and isolating the formed trehalose from the reaction system. Although the former facilitates the growth of microorganisms with a relative easiness, it requires a sequentially-complicated step for collecting trehalose from the microorganisms which contain at most 15 w/w % trehalose, on a dry solid basis (d.s.b.). While the latter enables the separation of trehalose itself with a relative easiness, but it is theoretically difficult to increase the trehalose yield by allowing enzymes to act on substrates at a considerably-high concentration because the enzymatic reaction *per se* is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

In view of the foregoing, the present inventors energetically screened enzymes which form saccharides having a trehalose structure from amylaceous saccharides, and found that microorganisms such as those of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36 produce an absolutely novel enzyme which forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of 3 or higher. Before or after this finding, it was revealed that such non-reducing saccharides are almost quantitatively hydrolyzed into trehalose and glucose and/or maltooligosaccharides by other enzymes produced from the same microorganisms of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36. Since the combination use of such enzymes enables to form a desired amount of trehalose with a relative easiness, the aforementioned objects relating to trehalose would be completely overcome. Insufficient producibility of such enzymes by the microorganisms results in a drawback that a relatively-large scale culture of the microorganisms is inevitable to industrially produce trehalose and/or non-reducing saccharides having a trehalose structure as an end unit.

Recombinant DNA technology has made a remarkable progress in recent years. At present, even an enzyme, whose total amino acid sequence has not yet been revealed, can be readily prepared in a desired amount, if a gene encoding the enzyme was once isolated and the base sequence was decoded, by preparing a recombinant DNA containing a DNA which encodes the enzyme, introducing the recombinant DNA into microorganisms or cells of plants or animals, and culturing the resultant transformants. Under these circumstances, urgently required are the finding of genes which encode these enzymes and the elucidation of their base sequences.

It is an aim of the present invention to provide a DNA which encodes an enzyme that releases trehalose from non-reducing saccharides having a trehalose structure as an end unit.

It is a further aim of the present invention to provide a replicable recombinant DNA containing the aforesaid DNA.

It is yet another aim of the present invention to provide a transformant which is prepared by introducing the recombinant DNA into an appropriate host.

It is a further aim of the present invention to prepare the aforesaid enzyme by the application of the recombinant DNA technology.

It is a further aim of the present invention to provide a preparation of the enzyme.

It is a further aim of the present invention to provide a method for converting non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

The first aim of the present invention is attained by a DNA which encodes an enzyme that releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glu-

cose polymerization of 3 or higher.

The second aim of the present invention is attained by a replicable recombinant DNA which contains the aforesaid DNA and a self-replicable vector.

The third aim of the present invention is attained by a transformant prepared by introducing the aforesaid self-replicable vector into an appropriate host.

The fourth aim of the present invention is attained by a recombinant enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

The fifth aim of the present invention is attained by a process to produce the recombinant enzyme comprising culturing a transformant capable of forming the enzyme in a nutrient culture medium, and recovering the formed enzyme from the resultant culture.

The sixth aim of the present invention is attained by a method for converting non-reducing saccharides containing a step of allowing the recombinant enzyme to act on non-reducing saccharides, having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, to release trehalose from the saccharides.

The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings, in which:

FIG. 1 shows the optimum temperature of an enzyme derived from *Rhizobium* sp. M-11.

FIG. 2 shows the optimum temperature of an enzyme derived from *Arthrobacter* sp. Q36.

FIG. 3 shows the optimum pH of an enzyme derived from *Rhizobium* sp. M-11.

FIG. 4 shows the optimum pH of an enzyme derived from *Arthrobacter* sp. Q36.

FIG. 5 shows the thermal stability of an enzyme derived from *Rhizobium* sp. M-11.

FIG. 6 shows the thermal stability of an enzyme derived from *Arthrobacter* sp. Q36.

FIG. 7 shows the pH stability of an enzyme derived from *Rhizobium* sp. M-11.

FIG. 8 shows the pH stability of an enzyme derived from *Arthrobacter* sp. Q36.

FIG. 9 shows the restriction map of the recombinant DNA pBMU27 according to the present invention. In the figure, the bold-lined part is a DNA encoding an enzyme derived from *Rhizobium* sp. M-11.

FIG. 10 shows the restriction map of the recombinant DNA pBRT32 according to the present invention. In the figure, the bold-lined part is a DNA encoding an enzyme derived from *Arthrobacter* sp. Q36.

The DNA according to the present invention exerts the production of the enzyme encoded by the DNA in a manner that the DNA is inserted into an appropriate self-replicable vector to form a replicable recombinant DNA, followed by introducing the recombinant DNA into a host, incapable of producing the enzyme per se but readily replicable, to form a transformant.

Although the recombinant DNA *per se* does not produce the enzyme, the production of the enzyme encoded by the DNA is attained by introducing the recombinant DNA into a host, incapable of producing the enzyme but replicable with a relative easiness, to form a transformant, and culturing the transformant to produce the enzyme.

The transformant according to the present invention produces the enzyme when cultured.

The recombinant enzyme according to the present invention releases trehalose when acts on non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

The recombinant enzyme is readily obtained in a desired amount by culturing the transformant according to the invention.

Non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher are converted into trehalose and glucose and/or maltooligosaccharides.

The present invention is based on the finding of a novel enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. Such an enzyme can be obtained from cultures of microorganisms of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36, and the present inventors isolated the enzyme by the combination use of conventional purification methods using column chromatography mainly, examined the properties and features, and revealed the reality, i.e. it is a polypeptide having the following physicochemical properties:

(1) Action

Releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher;

(2) Molecular weight

About 57,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE);

(3) Isoelectric point

About 3.3-4.6 on isoelectrophoresis;

(4) Optimum temperature

Exhibiting an optimum temperature of around 35-45°C when incubated at pH 7.0 for 30 min;

(5) Optimum pH

Exhibiting an optimum pH of around 6.0-7.5 when incubated at 40°C for 30 min;

(6) Thermal stability

Stable up to a temperature of around 30-45°C when incubated at pH 7.0 for 60 min; and

(7) pH Stability

Stable up to a pH of around 5.5-10.0 when incubated at 25°C for 16 hours.

Experiments, which were conducted to reveal the physicochemical properties of the enzymes produced by microorganisms of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36 (the enzymes from *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36 are respectively designated as "enzyme M-11" and "enzyme Q36" hereinafter), are explained in the below:

Experiment 1

Purification of enzyme

Experiment 1-1

Purification of enzyme M-11

In 500-ml Erlenmeyer flasks were placed 100 ml aliquots of a liquid culture medium (pH 7.0) containing 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate, and 0.1 w/v % potassium dihydrogen phosphate, and the flasks were autoclaved at 120°C for 20 min to effect sterilization. After cooling the flasks a seed culture of *Rhizobium* sp. M-11 was inoculated into each liquid culture medium in each flask, followed by the incubation at 27°C for 24 hours under rotary-shaking conditions. Twenty L of a fresh preparation of the same liquid culture medium was put in a 30-L jar fermentor and sterilized, followed by inoculating one v/v % of the culture obtained in the above into the sterilized liquid culture medium in the jar fermentor, and incubating it at a pH of 6-8 and 30°C for 24 hours under aeration-agitation conditions.

Thereafter, about 18 L of the resultant culture was subjected to an ultra-high pressure cell disrupting apparatus to disrupt cells. The resultant suspension was centrifuged to obtain a supernatant, and to about 16 L of which was added ammonium sulfate to give a 20 w/v % saturation, followed by the standing at 4°C for one hour and the centrifugation to remove sediment. To the resultant supernatant was added ammonium sulfate to give a 60 w/v % saturation, and the solution was allowed to stand at 4°C for 24 hours and centrifuged to collect sediment which was then dissolved in a minimum amount of 10 mM phosphate buffer (pH 7.0). The solution thus obtained was dialyzed against 10 mM phosphate buffer (pH 7.0) for 24 hours, and centrifuged to remove insoluble substances. The resultant supernatant was fed to a column packed with "DEAE-TOYOPEARL®", a product for ion-exchange chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM phosphate buffer (pH 7.0), followed by feeding to the column a linear gradient buffer of sodium chloride ranging from 0 M to 0.5 M in 10 mM phosphate buffer (pH 7.0). Fractions containing the objective enzyme were collected from the eluate, pooled, dialyzed for 10 hours against 50 mM phosphate buffer (pH 7.0) containing 2 M ammonium sulfate, and centrifuged to remove insoluble substances. Thereafter, the resultant supernatant was fed to a column, which had been packed with "BUTYL TOYOPEARL®", a gel for hydrophobic column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, and equilibrated with 50 mM phosphate buffer (pH 7.0) containing 2 M ammonium sulfate, followed by feeding to the column a linear gradient buffer of ammonium sulfate ranging from 2 M to 0 M in 50 mM phosphate buffer (pH 7.0). Fractions containing the objective enzyme were collected from the eluate, pooled, fed to a column packed with "TOYOPEARL® HW-55", a product for gel filtration column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 50 mM phosphate buffer (pH 7.0), followed by feeding to the column 50 mM phosphate buffer (pH 7.0) and collecting fractions containing the objective enzyme. The enzyme thus obtained had a specific activity of about 240 units/mg protein, and the yield was about 650 units per L of the culture.

Throughout the specification the enzyme activity is expressed by the value measured on the following assay: Place 4 ml of 50 mM phosphate buffer (pH 7.0) containing 1.25 w/v % maltotriose in a test tube, add one ml of an enzyme solution to the tube, and incubate the resultant solution at 40°C for 30 min to effect enzymatic reaction. Thereafter, one ml of the reaction mixture is mixed with 2 ml of copper reagent to suspend

the enzymatic reaction, followed by assaying the reducing activity by the Somogyi-Nelson's method. As a control, an enzyme, which has been previously inactivated by heating at 100°C for 10 min, is similarly treated as above. One unit activity of the enzyme is defined as the amount of enzyme which increases the reducing power corresponding to one  $\mu$ mol glucose per min under the above conditions.

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#### Experiment 1-2

#### Purification of enzyme Q36

10 Similarly as in Experiment 1-1, a seed culture of *Arthrobacter* sp.Q36 was cultured, and the resultant culture was treated to obtain a purified enzyme Q36 having a specific activity of about 450 units/mg protein in a yield of about 650 units per L of the culture.

#### Experiment 2

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#### Physicochemical property of enzyme

#### Experiment 2-1

#### Action

25 According to the method disclosed in Japanese Patent Application No.349,216/93, a non-reducing saccharide containing 98 w/w % or higher, d.s.b.,  $\alpha$ -glucosyltrehalose,  $\alpha$ -maltosyltrehalose,  $\alpha$ -maltotriosyltrehalose,  $\alpha$ -maltotetraosyltrehalose or  $\alpha$ -maltopentaosyltrehalose. Either of the non-reducing saccharides as a substrate was dissolved in 50 mM phosphate buffer (pH 7.0) into a 20 w/v % solution which was then mixed with 2 units/g substrate of the purified enzyme M-11 or Q36 in Experiment 1 and subjected to an enzymatic reaction at 40°C for 48 hours. The reaction mixture was desalted in usual manner, fed to "WB-T-330", a column for high-performance liquid chromatography (HPLC) commercialized by Wako Pure Chemical Industries, Ltd., Tokyo, Japan, followed by feeding to the column distilled water at a flow rate of 0.5 ml/min at ambient temperature to isolate saccharides contained in the reaction mixture while monitoring the saccharide concentration of the eluate with "MODEL RI-8012", a differential refractometer commercialized by Tosoh Corporation, Tokyo, Japan. As a control, an aqueous solution which contains either maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose was similarly treated as above, and the resultant mixture was analyzed. The saccharide compositions of the reaction mixtures were tabulated in Tables 1 and 2.

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Table 1

|    | Substrate                         | Saccharide in reaction mixture    | Saccharide composition (%) |
|----|-----------------------------------|-----------------------------------|----------------------------|
| 5  | $\alpha$ -Glucosyltrehalose       | Trehalose                         | 17.5                       |
|    |                                   | Glucose                           | 6.5                        |
|    |                                   | $\alpha$ -Glucosyltrehalose       | 76.0                       |
| 10 | $\alpha$ -Maltosyltrehalose       | Trehalose                         | 44.3                       |
|    |                                   | Maltose                           | 44.4                       |
|    |                                   | $\alpha$ -Maltosyltrehalose       | 11.3                       |
| 15 | $\alpha$ -Maltotriosyltrehalose   | Trehalose                         | 39.5                       |
|    |                                   | Maltotriose                       | 60.0                       |
|    |                                   | $\alpha$ -Maltotriosyltrehalose   | 0.5                        |
| 20 | $\alpha$ -Maltotetraosyltrehalose | Trehalose                         | 34.2                       |
|    |                                   | Maltotetraose                     | 65.5                       |
|    |                                   | $\alpha$ -Maltotetraosyltrehalose | 0.3                        |
| 25 | $\alpha$ -Maltopentaosyltrehalose | Trehalose                         | 29.1                       |
|    |                                   | Maltopentaose                     | 70.6                       |
|    |                                   | $\alpha$ -Maltopentaosyltrehalose | 0.3                        |
| 30 | Maltotriose                       | Maltotriose                       | 100.0                      |
|    | Maltotetraose                     | Maltotetraose                     | 100.0                      |
|    | Maltopentaose                     | Maltopentaose                     | 100.0                      |
| 35 | Maltohexaose                      | Maltohexaose                      | 100.0                      |
|    | Maltoheptaose                     | Maltoheptaose                     | 100.0                      |

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Table 2

|    | Substrate                         | Saccharide in reaction mixture    | Saccharide composition (%) |
|----|-----------------------------------|-----------------------------------|----------------------------|
| 5  | $\alpha$ -Glucosyltrehalose       | Trehalose                         | 19.3                       |
|    |                                   | Glucose                           | 10.2                       |
|    |                                   | $\alpha$ -Glucosyltrehalose       | 70.5                       |
| 10 | $\alpha$ -Maltosyltrehalose       | Trehalose                         | 44.5                       |
|    |                                   | Maltose                           | 44.4                       |
|    |                                   | $\alpha$ -Maltosyltrehalose       | 11.1                       |
| 15 | $\alpha$ -Maltotriosyltrehalose   | Trehalose                         | 38.8                       |
|    |                                   | Maltotriose                       | 60.7                       |
|    |                                   | $\alpha$ -Maltotriosyltrehalose   | 0.5                        |
| 20 | $\alpha$ -Maltotetraosyltrehalose | Trehalose                         | 34.1                       |
|    |                                   | Maltotetraose                     | 65.7                       |
|    |                                   | $\alpha$ -Maltotetraosyltrehalose | 0.2                        |
| 25 | $\alpha$ -Maltopentaosyltrehalose | Trehalose                         | 29.3                       |
|    |                                   | Maltopentaose                     | 70.4                       |
|    |                                   | $\alpha$ -Maltopentaosyltrehalose | 0.3                        |
| 30 | Maltotriose                       | Maltotriose                       | 100.0                      |
|    | Maltotetraose                     | Maltotetraose                     | 100.0                      |
|    | Maltopentaose                     | Maltopentaose                     | 100.0                      |
| 35 | Maltohexaose                      | Maltohexaose                      | 100.0                      |
|    | Maltoheptaose                     | Maltoheptaose                     | 100.0                      |

As shown in Tables 1 and 2, enzymes M-11 and Q36 almost quantitatively released trehalose, glucose and maltooligosaccharides from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. These enzymes did not act on maltooligosaccharides, as a substrate, having a degree of glucose polymerization of 3 or higher. These facts indicate that these enzymes selectively act on non-reducing saccharides having a trehalose structure as an end unit and having a degree of polymerization degree of 3 or higher, and specifically hydrolyze the glycosidic bond between trehalose- and glycosyl-residues. Such an enzyme has never been reported and is estimated to have a novel enzymatic reaction mechanism.

#### Experiment 2-2

##### Molecular weight

In accordance with the method reported by U. K. Laemmli in *Nature*, Vol.227, pp.680-685 (1970), the purified enzymes M-11 and Q36 in Experiment 1 were respectively electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis to show a single protein band at a position corresponding to about 57,000-68,000 daltons. The marker proteins used in this experiment were myosin (MW=200,000 daltons),  $\beta$ -galactosidase (MW=116,250 daltons), phosphorylase B (MW=97,400 daltons), serum albumin (MW=66,200 daltons) and ovalbumin (MW=45,000 daltons).

## Experiment 2-3

### Isoelectric point

5 The purified enzymes M-11 and Q36 obtained in Experiment 1 gave an isoelectric point of about 3.3-4.6 on isoelectrophoresis.

## Experiment 2-4

### Optimum temperature

The optimum temperature of the purified enzymes M-11 and Q36 obtained in Experiment 1 was about 35-45°C as shown in FIGs. 1 and 2 when incubated in usual manner in 50 mM phosphate buffer (pH 7.0) for 30 min.

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## Experiment 2-5

### Optimum pH

20 The optimum pH of the purified enzymes M-11 and Q36 obtained in Experiment 1 was about 6.0-7.5 as shown in FIGs. 3 and 4 when experimented in usual manner by incubating them at 40°C for 30 min in 50 mM acetate buffer, phosphate buffer or sodium carbonate-sodium hydrogen carbonate buffer having different pHs.

## Experiment 2-6

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### Thermal stability

The purified enzymes M-11 and Q36 obtained in Experiment 1 were stable up to a temperature of about 30-45°C as shown in FIGs. 5 and 6 when experimented in usual manner by incubating them in 50 mM phosphate buffer (pH 7.0) for 60 min.

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## Experiment 2-7

### pH Stability

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The purified enzymes M-11 and Q36 obtained in Experiment 1 were stable up to a pH of about 5.5-10.0 as shown in FIGs. 7 and 8 when experimented in usual manner by incubating them at 25°C for 16 hours in 50 mM acetate buffer, phosphate buffer or sodium carbonate-sodium hydrogen carbonate buffer having different pHs.

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## Experiment 2-8

### Amino acid sequence containing the N-terminal

45 The amino acid sequence containing the N-terminal of the purified enzyme M-11 obtained in Experiment 1 was analyzed on "MODEL 470A", a gas-phase protein sequencer commercialized by Applied Biosystems, Inc., Foster City, USA, to reveal that it has the amino acid sequence as shown in SEQ ID NO:5.

The amino acid sequence containing the N-terminal of the purified enzyme Q36 was analyzed similarly as above to reveal that it has the amino acid sequence as shown in SEQ ID NO:6.

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## Experiment 2-9

### Partial amino acid sequence

55 An adequate amount of the purified enzyme M-11 obtained in Experiment 1-1 was weighed, dialyzed against 10 mM Tris-HCl buffer (pH 9.0) at 4°C for 18 hours, and admixed with 10 mM Tris-HCl buffer (pH 9.0) to give a concentration of about one mg/ml of the enzyme. About one ml of the resultant solution was placed in a container, admixed with 10 µg lysyl endopeptidase, and incubated at 30°C for 22 hours to partially hydrolyze



the enzyme. The resultant hydrolysate was applied to "CAPCELL-PAK C18", a column for reverse-phase high-performance liquid chromatography commercialized by Shiseido Co., Ltd., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % trifluoroacetate containing 16 v/v % aqueous acetonitrile, followed by feeding to the column 0.1 v/v % trifluoroacetate at a flow rate of 0.9 ml/min while increasing the concentration of acetonitrile from 16 v/v % to 64 v/v % to separately collect fractions containing a peptide fragment eluted about 43 min or about 57 min after the initiation of feeding (the peptide fragments were respectively named "peptide fragment A" and "peptide fragment B"). Fractions containing the peptide fragment A or B were separately pooled, dried *in vacuo*, and dissolved in 0.1 v/v % trifluoroacetate containing 50 v/v % aqueous acetonitrile. Similarly as in Experiment 2-8, the peptide fragments A and B were analyzed to reveal that they have the amino acid sequences as shown in SEQ ID NOs:7 and 8, respectively.

Similarly as in enzyme M-11, enzyme Q36 obtained in Experiment 1-2 was partially hydrolyzed, and the resultant was fed to "μBONDAPAK C18", a column for reverse-phase high-performance liquid chromatography commercialized by Japan Millipore Ltd., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % trifluoroacetate containing 24 v/v % aqueous acetonitrile, followed by feeding to the column 0.1 v/v % trifluoroacetate containing 24 v/v % aqueous acetonitrile while increasing the concentration of aqueous acetonitrile from 24 v/v % to 44 v/v % at a flow rate of 0.9 ml/ml. Fractions containing a peptide fragment eluted about 4 min or about 24 min after the initiation of feeding (the fractions were respectively called "peptide fragment C" and "peptide fragment D" hereinafter) were respectively collected, pooled, dried *in vacuo*, and dissolved in 0.1 v/v % trifluoroacetate containing 50 v/v % aqueous acetonitrile. Analyses of the peptide fragments C and D conducted similarly as above have revealed that they have amino acid sequences as shown in SEQ ID NOs:9 10 respectively.

No enzyme having these physicochemical properties has been known, and this concluded that it is a novel substance. Referring to *Rhizobium* sp. M-11, it is a microorganism which was isolated from a soil of Okayama-city, Okayama, Japan, deposited on December 24, 1992, in National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Tsukuba, Ibaraki, Japan, and accepted under the accession number of FERM BP-4130, and it has been maintained by the institute. *Arthrobacter* sp. Q36 is a microorganism which was isolated from a soil of Soja-city, Okayama, Japan, deposited on June 3, 1993, in the same institute, and accepted under the accession number of FERM BP-4316, and it has been maintained by the institute. Japanese Patent Application No.340,343/93, applied by the same applicant, discloses the properties and features of the non-reducing saccharide-forming enzyme as well as the detailed bacteriological properties of these microorganisms.

The present inventors energetically screened the chromosomal DNA of *Rhizobium* sp. M-11 by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequence of enzyme M-11 as revealed in Experiment 2-8 or 2-9, and obtained a DNA fragment which consists of 1,767 base pairs having the base sequence as shown in the following SEQ ID NO:1 that initiates from the 5'-terminus. The decoding of the base sequence of the enzyme has revealed that it has an amino acid sequence consisting of 589 amino acids as shown in SEQ ID NO:2.

Similarly as in enzyme M-11, the chromosomal DNA of enzyme Q36 was screened by using an oligonucleotide as a probe which had been chemically synthesized based on a partial amino acid sequence of enzyme Q36, and this yielded a DNA fragment having a base sequence consisting of 1,791 base pairs as shown in SEQ ID NO:3. The base sequence was decoded to reveal that enzyme Q36 has an amino acid sequence consisting of 597 amino acids as shown in SEQ ID NO:4.

The sequential experimental steps used for revealing the base sequence and amino acid sequence as shown in SEQ ID NOs:1 to 4 are summarized as below:

- (1) The enzyme was isolated from a culture of a donor microorganism and highly purified. The purified enzyme was partially hydrolyzed with protease, and the resultant 2 different types of peptide fragments were isolated and determined their amino acid sequences;
- (2) Separately, a chromosomal DNA was isolated from a donor microorganism's cell, purified and partially digested by a restriction enzyme to obtain a DNA fragment consisting of about 2,000-6,000 base pairs. The DNA fragment was ligated by DNA ligase to a plasmid vector, which had been previously cut with a restriction enzyme, to obtain a recombinant DNA;
- (3) The recombinant DNA was introduced into *Escherichia coli* to obtain transformants, and from which an objective transformant containing a DNA encoding the enzyme was selected by the colony hybridization method using an oligonucleotide, as a probe, which had been chemically synthesized based on the afore-said partial amino acid sequence; and
- (4) The recombinant DNA was obtained from the selected transformant and annealed with a primer, followed by allowing a DNA polymerase to act on the resultant to extend the primer, and determining the base sequence of the resultant complementary chain DNA by the dideoxy chain termination method. The com-

parison of an amino acid sequence, estimable from the determined base sequence with the aforesaid amino acid sequence, confirmed that the base sequence encodes the enzyme.

The recombinant enzyme as referred to in the specification mean the whole recombinant enzymes which are preparable by the recombinant DNA technology and capable of releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. Generally, the recombinant enzyme according to the present invention has a revealed amino acid sequence, and, as an example, the amino acid sequence as shown in SEQ ID NO:2 or 4 which initiates from the N-terminal, as well as homologous ones to it, can be mentioned. Variants having amino acid sequences homologous to the one as shown in SEQ ID NO:2 or 4 can be obtained by replacing one or more bases in SEQ ID NO:2 or 4 with other bases without substantially alternating the inherent activity of the enzyme. Although even when used the same DNA and it also depends on hosts into which the DNA is introduced, as well as on ingredients and components of nutrient culture media used for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have amino acid sequences similar to that of SEQ ID NO:2 or 4, as well as having the enzymatic activity inherent to the enzyme encoded by the DNA but defective one or more amino acids located near to the N-terminal of the amino acid sequence of SEQ ID NO:2 or 4 and/or having one or more amino acids newly added to the N-terminal by the modification of intracellular enzymes of hosts after the DNA expression. In view of the technical background in the art, the enzyme as referred to in the present invention includes those which have the amino acid sequence corresponding to that of SEQ ID NO:2 or 4, and those which substantially have the amino acid sequence as shown in SEQ ID NO:2 or 4 except that one or more amino acids in the amino acid sequence are defected, newly added to or replaced with other amino acids, as long as they release trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

In this field, it is known that one or more bases in DNAs can be replaced with other bases by the degeneracy of genetic code without alternating the amino acid sequences encoded by the DNAs. Based on this the DNA according to the present invention includes DNAs which contain the amino acid sequence of SEQ ID NO:1 or 3 and other DNAs, wherein one or more bases are replaced with other bases by degeneracy of genetic code, as long as they encode enzymes having the amino acid sequence as shown in SEQ ID NO:2 or 4 and homologous variants thereof.

According to the today's recombinant DNA technology, the determination of base sequences from the 5'-termini of DNAs define their complementary base sequences. Therefore, the DNA according to the present invention also includes complementary base sequences corresponding to any one of the aforesaid base sequences. Needless to say, one or more bases in the base sequence, which encodes the enzyme or their variants, can be readily replaced with other bases to allow the DNA to actually express the enzyme production in hosts.

The DNA according to the present invention is as described above, and any DNA derived from natural resources and those artificially synthesized can be used in the present invention as long as they have the aforementioned base sequences. The natural resources of the DNA according to the present invention are, for example, microorganisms of the genera *Rhizobium*, *Arthrobacter*, *Brevibacterium* and *Micrococcus*, i.e. *Rhizobium* sp. M-11 (FERM BP-4130), *Arthrobacter* sp. Q36 (FERM BP-4316), *Brevibacterium helvolum* (ATCC 11822) and *Micrococcus roseus* (ATCC 186) from which genes containing the present DNA can be obtained. These microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells were collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or  $\beta$ -glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, in the case of treating the cells with ultrasonication, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or treated with freezing- and thawing-methods. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment used in general in the art.

To artificially synthesize the DNA according to the present invention, it can be chemically synthesized by using the base sequence as shown in SEQ ID NO:1 or 3, or can be obtained in plasmid form by inserting a DNA, which encodes the amino acid sequence as shown in SEQ ID NO:2 or 4, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the DNA from the cells.

The present invention further relates to replicable recombinant DNAs which express the production of the enzyme according to the invention when introduced into microorganisms as well as plant- and animal-cells which do not produce the enzyme inherently but are readily proliferative. Such a recombinant DNA, which generally contains the aforesaid DNA and a self-replicable vector, can be prepared by conventional method with

a relative easiness when the material DNA is in hand. Examples of such a vector are plasmid vectors such as pBR322, pUC18, Bluescript II SK(+), pUB110, pTZ4, pC194, pHV14, TRp7, TEp7, pBS7, etc.; and phage vectors such as  $\lambda$ gt $\lambda$ C,  $\lambda$ gt $\lambda$ B,  $\phi$ 11,  $\phi$ 1,  $\phi$ 105, etc. Among these plasmid- and phage-vectors, pBR322, pUC18, Bluescript II SK(+),  $\lambda$ gt $\lambda$ C and  $\lambda$ gt $\lambda$ B are satisfactorily used in case that the present DNA should be expressed in *Escherichia coli*, while pUB110, pTZ4, pC194,  $\phi$ 11,  $\phi$ 1 and  $\phi$ 105 are satisfactorily used to express the DNA in microorganisms of the genus *Bacillus*. The plasmid vectors pHV14, TRp7, TEp7 and pBS7 are suitably used when the recombinant DNA is allowed to grow in 2 or more hosts.

The methods used to insert the present DNA into such vectors in the present invention may be conventional ones generally used in this field. A gene containing the present DNA and a self-replicable vector are first digested by a restriction enzyme and/or ultrasonic disintegrator, then the resultant DNA fragments and vector fragments are ligated. To digest DNAs and vectors, restriction enzymes which specifically act on nucleotides, particularly, type II restriction enzymes, more particularly, *Sau* 3A I, *Eco* R I, *Hind* III, *Bam* HI, *Sal* I, *Xba* I, *Sac* I, *Pst* I, etc., facilitate the ligation of the DNA fragments and vector fragments. The ligation of the DNA fragments and vector fragments is effected by annealing them first if necessary, then subjected to the action of a DNA ligase *in vivo* or *in vitro*. The recombinant DNA thus obtained is replicable without substantial limitation by introducing it into appropriate hosts, and culturing the resultant transformants.

The recombinant DNA according to the present invention can be introduced into appropriate host microorganisms including *Escherichia coli* and those of the genus *Bacillus* as well as actinomyces and yeasts. In the case of using *Escherichia coli* as a host, it can be cultured in the presence of the recombinant DNA and calcium ion, while in the case of using the microorganisms of the genus *Bacillus* the competent cell method and the colony hybridization method can be employed. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, and selecting the objective transformants which release trehalose from the non-reducing saccharides.

The transformants thus obtained extracellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with a small amount of amino acids and vitamins can be used as the nutrient culture media. Examples of the carbon sources are saccharides such as starch, starch hydrollysate, glucose, fructose and sucrose. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia, ammonium salts, urea, nitrate, peptone, yeast extract, defatted soy bean, corn steep liquor and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 25-65°C and a pH of 2-8 for about 1-6 days under aerobic aeration-agitation conditions. Such a culture can be used intact as an enzyme preparation, and, usually, it may be disrupted with ultrasonic disintegrator and/or cell-wall lysis enzymes prior to use, followed by separating the enzyme from the intact cells and cell debris by filtration and/or centrifugation, and purifying the enzyme. The methods used for purifying the enzyme in the invention include conventional ones in general. From cultures the intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separately sedimentation, gel filtration chromatography, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

As is described above, the enzyme exerts a distinct activity of forming trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, and such an activity has not yet been found in any conventional enzymes. Therefore, the use of the enzyme facilitates the preparation of trehalose in a relatively-high yield and efficiency from non-reducing saccharides such as  $\alpha$ -glucosyltrehalose,  $\alpha$ -maltosyltrehalose,  $\alpha$ -maltotriosyltrehalose,  $\alpha$ -maltotetraosyltrehalose and  $\alpha$ -maltopentaosyltrehalose in a considerably-high yield. These non-reducing saccharides can be obtained in a satisfactorily-high yield from starch hydrolysates, which are obtained by treating amylaceous substances such as starch, amylose and amylopectin prepared with acids and/or amylases, by using non-reducing saccharide-forming enzyme as disclosed in Japanese Patent Application No.349,216/93. Thus, trehalose, whose industrial preparation has been difficult, can be prepared from starch and amylaceous substances as a material with a relative easiness and in a desired amount when the present enzyme and the non-reducing saccharide-forming enzyme, as disclosed in Japanese Patent Application No.349,216/93, are used in combination.

As described in "Handbook of Amylases and Related Enzymes", 1st edition, edited by The Amylase Research Society of Japan, published by Pergamon Press plc, Oxford, England (1988),  $\alpha$ -amylase, maltotetraose-forming amylase, maltopentaose-forming amylase and maltohexaose-forming amylase are especially useful to prepare the reducing amylaceous saccharides used in the invention, and, the use of any one of these

amylases readily yields amylaceous saccharide mixtures rich in reducing amylaceous saccharides having a degree of glucose polymerization of 3 or higher in a considerably-high yield. If necessary, the combination use of such an amylase and a starch debranching enzyme such as pullulanase or isoamylase can increase the yield of the reducing amylaceous saccharides usable as a substrate for the non-reducing saccharide-forming enzyme, i.e. the non-reducing saccharides can be obtained by coexisting the non-reducing saccharide-forming enzyme in an aqueous solution containing as a substrate one or more of the reducing amylaceous saccharides in an amount up to a concentration of 50 w/v %, and subjecting the solution to an enzymatic reaction at a temperature of about 40-55°C and a pH of about 6-8 until a desired amount of the objective non-reducing saccharides are formed.

Usually, in the present conversion method, the recombinant enzyme according to the present invention is allowed to coexist in the aforesaid aqueous solution containing one or more of the non-reducing amylaceous saccharides, and to enzymatically react with the saccharides while keeping at a prescribed temperature and pH until a desired amount of trehalose is released.

Although the enzymatic reaction proceeds even below a concentration of 0.1 w/v % of a substrate, a higher concentration of 2 w/v %, preferably, 5-50 w/v % of a substrate can be satisfactorily used to apply the present conversion method to an industrial-scale production. The temperature and pH used in the enzymatic reaction are set within the ranges of which do not inactivate the recombinant enzyme and allow the recombinant enzyme to effectively act on substrates, i.e. a temperature up to about 55°C, preferably, a temperature in the range of about 40-55°C, and a pH of 5-10, preferably, a pH in the range of about 6-8. The amount and reaction time of the present recombinant enzyme are chosen dependently on the enzymatic reaction conditions. The enzymatic reaction effectively converts non-reducing saccharides into saccharide compositions containing trehalose and glucose and/or maltooligosaccharides, and, in the case of using  $\alpha$ -maltotriosyltrehalose as a substrate, the conversion rate reaches to approximately 100%. In the case of simultaneously subjecting starch hydrolysates to the action of either of the above amylases together with the non-reducing saccharide-forming enzyme and the present recombinant enzyme, non-reducing saccharides are formed from the hydrolysates while hydrolyzed into glucose and/or maltooligosaccharides, and because of this saccharide compositions with a relatively-high trehalose content can be effectively obtained in a relatively-high yield.

The reaction products obtained by the present conversion reaction can be used intact, and, usually, they are purified prior to use. Insoluble substances are eliminated from the reaction products by filtration and centrifugation, and the resultant solutions are decolorized with activated charcoal, desalted and purified on ion exchangers, and concentrated into syrupy products. Dependently on their use, the syrupy products are dried in vacuo and spray-dried into solid products. In order to obtain products which substantially consist of non-reducing saccharides, the above mentioned syrupy products are subjected to one or more methods such as chromatography using an ion exchanger, activated charcoal and silica gel to separate saccharides, separately sedimentation using alcohol and/or acetone, membrane filtration, fermentation by yeasts, and removal and decomposition of reducing saccharides by alkalis. The methods to treat a large amount of reaction mixture are, for example, fixed bed- or pseudomoving bed-ion exchange column chromatography as disclosed in Japanese Patent Laid-Open Nos.23,799/83 and 72,598/83, and such a method enables an effective industrial-scale production of products with a relatively-high trehalose content.

These trehalose and compositions containing the same have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant in food products in general, cosmetics and pharmaceuticals.

The following examples explain the present invention in more detail, and the techniques themselves used in the examples are conventional ones in this field, for example, those described by J. Sumbruck et al. in "Molecular Cloning A Laboratory Manual", 2nd edition, published by Cold Spring Harbor Laboratory Press (1989).

#### Example 1

##### Preparation of recombinant DNA containing DNA encoding enzyme M-11 and transformant

#### Example 1-1

##### Preparation of chromosomal DNA

A seed culture of *Rhizobium* sp. M-11 was inoculated into bacto nutrient broth medium (pH 7.0), and cultured at 27°C for 24 hours with a rotary shaker. The cells were separated from the resultant culture by centrifugation, suspended in TES buffer (pH 8.0), admixed with 0.05 w/v % lysozyme, and incubated at 37°C for 30

min. The resultant was freezed at -80°C for one hour, admixed with TSS buffer (pH 9.0), heated to 60°C, and further admixed with a mixture solution of TES buffer and phenol, and the resultant solution was chilled with ice, followed by centrifugally collecting the precipitated crude chromosomal DNA. To the supernatant was added 2 fold volumes of cold ethanol, and the re-precipitated crude chromosomal DNA was collected, suspended in SSC buffer (pH 7.1), admixed with 7.5 µg ribonuclease and 125 µg protease, and incubated at 37°C for one hour. Thereafter, a mixture solution of chloroform and isoamyl alcohol was added to the reaction mixture to extract the objective chromosomal DNA, and admixed with cold ethanol, followed by collecting the formed sediment containing the chromosomal DNA. The purified chromosomal DNA thus obtained was dissolved in SSC buffer (pH 7.1) to give a concentration of about one mg/ml, and the resultant solution was freezed at -80°C.

#### Example 1-2

##### Preparation of recombinant DNA pBMU27 and transformant BMU27

About one ml of the purified chromosomal DNA obtained in Example 1-1 was placed in a container, admixed with about 35 units of *Sau* 3AI, a restriction enzyme, and enzymatically reacted at 37°C for about 20 min to partially digest the chromosomal DNA, followed by recovering a DNA fragment consisting of about 2,000-6,000 base pairs by means of sucrose density-gradient ultracentrifugation. One µg of Bluescript II SK(+), a plasmid vector, was provided, subjected to the action of *Bam* HI, a restriction enzyme, to completely digest the plasmid vector, admixed with 10 µg of the DNA fragment and 2 units of T4 DNA ligase, and allowed to stand at 4°C overnight to ligate the DNA fragment to the vector fragment. To the resultant recombinant DNA was added 30 µl of "Epicurian Coli® XLI-Blue", competent cell commercialized by Toyobo Co., Ltd., Tokyo, Japan, allowed to stand under ice-chilling conditions for 30 min, heated to 42°C, admixed with SOC broth, and incubated at 37°C for one hour to introduce the recombinant DNA into *Escherichia coli*.

The resultant transformant was inoculated into agar plate (pH 7.0) containing 50 µg/ml of 5-bromo-4-chloro-3-indolyl-β-galactoside, and cultured at 37°C for 18 hours, followed by placing a nylon film on the agar plate to fix thereon about 6,000 colonies formed on the agar plate. Based on the amino acid sequence located at positions from 8 to 13 as shown in SEQ ID NO:7, i.e. Phe-Asp-Ile-Trp-Ala-Pro, the base sequence of probe 1 represented by 5'-TTYGAYATHTGGGCNCC-3' was chemically synthesized, labelled with <sup>32</sup>P, and hybridized with the colonies of transformants fixed on the nylon film, followed by selecting 14 transformants which exhibited a strong hybridization.

The objective recombinant DNA was selected in usual manner from the 14 transformants, and, in accordance with the method described by E. M. Southern in *Journal of Molecular Biology*, Vol.98, pp.503-517 (1975), the recombinant DNA was hybridized with probe 2 having the base sequence as shown in SEQ ID NO:8, which had been chemically synthesized based on the amino acid sequence located at positions from 2 to 6, i.e. Asp-Trp-Ala-Glu-Ala, in SEQ ID NO:8, followed by selecting a recombinant DNA strongly hybridized with the probe 2. The recombinant DNA and transformant thus selected were respectively named "pBMU27" and "BMU27".

The transformant BMU27 was inoculated into L-broth (pH 7.0) containing 100 µg/ml ampicillin, and cultured at 37°C for 24 hours by a rotary shaker. After completion of the culture, the resultant cells were collected from the culture by centrifugation, and treated with the alkaline method in general to extracellularly extract a recombinant DNA. The extract was in usual manner purified and analyzed to reveal that the recombinant DNA pBMU27 consists of about 5,700 base pairs and has the structure expressed by the restriction map as shown in FIG. 9. It was found that, as shown in FIG. 9, the DNA which consists of 1,767 base pairs for encoding the enzyme M-11 is positioned in the downstream near to the digested site of *Eco* RV, a restriction enzyme.

#### Example 1-3

##### Production of enzyme by transformant BMU27

A liquid nutrient culture medium consisting of 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate was adjusted to pH 7.0, admixed with 50 µg/ml ampicillin, autoclaved at 120°C for 20 min, cooled and inoculated with a seed culture of transformant BMU27 obtained in Example 1-2, followed by culturing the transformant at 37°C for 24 hours by a rotary shaker. The resultant culture was treated with ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances. The supernatant thus obtained was assayed for the enzyme activity to find that one L of the culture yielded about 4,000 units of the enzyme.

As a control, a seed culture of *Escherichia coli* XLI-Blue or *Rhizobium* sp. M-11 was inoculated in the same

fresh preparation of the same liquid nutrient culture medium but free of ampicillin, and, in the case of culturing *Rhizobium* sp. M-11, it was cultured and treated similarly as above except that the cultivation temperature was set to 30°C. Assaying the resultant activity, one L culture of *Rhizobium* sp. M-11 yielded about 2,000 units of the enzyme, and the yield was significantly lower than that of transformant BMU27. *Escherichia coli* XLI-Blue used as a host did not form the enzyme.

Thereafter, the enzyme produced by the transformant MBU27 was purified similarly as in Experiment 1-1, and examined on the properties and characters. As a result, it was revealed that it has substantially the same physicochemical properties as enzyme M-11, i.e. it has a molecular weight of about 57,000-68,000 daltons on SDS-PAGE and an isoelectric point of about 3.3-4.6 on isoelectrophoresis. The results indicate that the present enzyme can be prepared by the recombinant DNA technology, and the yield can be significantly increased thereby.

### Example 2

#### Preparation of complementary chain DNA derived from *Rhizobium* sp. M-11, and determination for its base sequence and amino acid sequence

Two µg of the recombinant DNA pBMU27 obtained in Example 1-2 was provided, admixed with 2 M aqueous sodium hydroxide solution to effect degeneration, and admixed with an adequate amount of cold ethanol, followed by collecting the formed sediment containing a template DNA and drying the sediment *in vacuo*. To the template DNA were added 50 pmole/ml of a chemically synthesized primer 1 represented by 5'-GTAAAC-GACGGCCAGT-3', 10 µl of 40 mM Tris-HCl buffer (pH 7.5) containing 20 mM magnesium chloride and 20 mM sodium chloride, and the mixture was incubated at 65°C for 2 min to effect annealing and admixed with 2 µl of an aqueous solution containing dATP, dGTP and dTTP in respective amounts of 7.5 µM, 0.5 µl of [ $\alpha$ -<sup>32</sup>P]dCTP (2 mCi/ml), one µl of 0.1 M dithiothreitol, and 2 µl of 1.5 units/ml T7 DNA polymerase, followed by incubating the resultant mixture at 25°C for 5 min to extend the primer 1 from the 5'-terminus to the 3'-terminus. Thus, a complementary chain DNA was formed.

The reaction product containing the complementary chain DNA was divided into quarters, to each of which 2.5 µl of 50 mM aqueous sodium chloride solution containing 80 µM dNTP and 8 µM ddATP, ddCTP, ddGTP or ddTTP was added, and the resultant mixture was incubated at 37°C for 5 min, followed by suspending the reaction by the addition of 4 µl of 98 v/v % aqueous formamide solution containing 20 mM EDTA, 0.05 w/v % bromophenol blue, and 0.05 w/v % xylene cyanol. The reaction mixture was heated with a boiling-water bath for 3 min, and a portion of which was placed on a gel containing 6 w/v % polyacrylamide, and electrophoresed by energizing the gel with a constant voltage of about 2,000 volts to separate DNA fragments, followed by fixing the gel in usual manner, drying the gel and subjecting the resultant gel to autoradiography.

Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of about 2,161 base pairs as shown in SEQ ID NO:11. An amino acid sequence estimable from the base sequence was as shown in SEQ ID NO:11 and was compared with the amino acid sequence containing the N-terminal or the partial amino acid sequence of enzyme M-11 as shown in SEQ ID NO:5, 7 or 8. As a result, it was found that the amino acid sequence containing the N-terminal of SEQ ID NO:5 corresponds to the amino acid sequence located at positions from 8 to 27 in SEQ ID NO:11, and the partial amino acid sequence of SEQ ID NO:7 or 8 corresponds to the amino acid sequence located at positions from 10 to 30 or at positions from 493 to 509 in SEQ ID NO:11. These results indicate that enzyme M-11 has the amino acid sequence of SEQ ID NO:2, and it is encoded by the DNA having the base sequence as shown in SEQ ID NO:1.

### Example 3

#### Preparation of recombinant DNA containing DNA derived from *Arthrobacter* sp. Q36, and transformant

##### Example 3-1

#### Preparation of chromosomal DNA

Similarly as in Example 1-1, a chromosomal DNA was isolated from *Arthrobacter* sp. Q36, purified and dissolved in SSC buffer (pH 7.1) to give a concentration of about one mg/ml, and the resultant solution was freezed at -80°C for storage.

Example 3-2Preparation of recombinant DNA pBRT32 and transformant BRT32

The purified chromosomal DNA obtained in Example 3-1 was partially digested similarly as in Example 1-2, followed by recovering a DNA fragment consisting of about 2,000-6,000 base pairs by sucrose density gradient ultracentrifugation. The DNA fragment was ligated to a lysate of Bluescript II SK(+) which had been treated with *Bam* HI, and the resultant recombinant DNA was introduced into *Escherichia coli* XLI-Blue. The transformants thus obtained were cultured similarly as in Example 1-2 on agar plates containing 5-bromo-4-chloro-3-indolyl- $\beta$ -galactoside, and the formed about 5,000 colonies were fixed on a nylon film, while the probe 3 represented by 5'-ATGGGNTGGGAYCCNGC-3' was chemically synthesized based on the amino acid sequence of Met-Gly-Trp-Asp-Pro-Ala located at positions from 5 to 10 in SEQ ID NO:9, labelled with  $^{32}$ P, and hybridized with transformant colonies which had been fixed on the nylon film, followed by selecting 10 transformants which strongly hybridized with the probe 3.

Similarly as in Example 1-2, the objective recombinant DNA was selected from 10 transformants, and hybridized with probe 4 represented by 5'-TAYGAYGTNTGGGC-3' which had been chemically synthesized based on the amino acid sequence of Tyr-Asp-Val-Trp-Ala located at positions from 8 to 12 in SEQ ID NO:10, followed by selecting a recombinant DNA which strongly hybridized with probe 4. The recombinant DNA and transformant thus selected were respectively named "pBRT32" and "BRT32".

The transformant BRT32 was inoculated into L-broth containing ampicillin, and cultured similarly as in Example 1-2, and the proliferated cells were collected from the resultant culture, and from which a recombinant DNA was extracted, purified and analyzed to reveal that the recombinant DNA pBRT32 consists of about 6,200 base pairs and has the structure of the restriction map as shown in FIG. 10. As shown in FIG. 10, it was revealed that the DNA, which consists of 1,791 base pairs for encoding the DNA of enzyme Q36, is located in the downstream near to the cleavage site of *Kpn* I.

Example 3-3Production of enzyme by transformant BRT32

A liquid nutrient culture medium consisting of 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate was adjusted to pH 7.0, admixed with 50  $\mu$ g/ml ampicillin, autoclaved at 120°C for 20 min, cooled and inoculated with a seed culture of the transformant BRT32 obtained in Example 3-2, followed by culturing the transformant at 37°C for 24 hours by a rotary shaker. The resultant culture was treated with an ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances. The supernatant thus obtained was assayed for the present enzyme activity to find that one L of the culture yielded about 3,900 units of the enzyme.

As a control, a seed culture of *Escherichia coli* XLI-Blue or *Arthrobacter* sp. Q36 was inoculated into a fresh preparation of the same liquid nutrient culture medium but free of ampicillin, and, in the case of culturing *Arthrobacter* sp. Q36, it was cultured and treated similarly as above except that the cultivation temperature was set to 30°C. Assaying the enzyme activity, one L of the culture of *Arthrobacter* sp. Q36 yielded about 1,800 units of the enzyme, and the yield was significantly lower than that of the transformant BRT32. The *Escherichia coli* XLI-Blue used as a host did not form the enzyme.

Thereafter, the enzyme produced by the transformant BRT32 was purified similarly as in Experiment 1-1, and examined on the properties and characters to reveal that it has substantially the same physicochemical properties as that of enzyme Q36, i.e. it has a molecular weight of about 57,000-68,000 daltons on SDS-PAGE and an isoelectric point of about 3.3-4.6 on isoelectrophoresis. These results indicate that the enzyme can be prepared by the recombinant DNA technology, and the yield can be significantly increased thereby.

Example 4Preparation of complementary chain DNA derived from *Arthrobacter* sp. Q36, and determination for its base sequence and amino acid sequence

The recombinant DNA pBRT32 obtained in Example 3-2 was similarly treated as in Example 2 to form a template DNA which was then annealed together with the primer 1, followed by allowing T7 DNA polymerase to act on the resultant to extend the primer 1 from the 5'-terminus to the 3'-terminus to obtain a complementary

chain DNA. Similarly as in Example 2, the complementary chain DNA was subjected to the dideoxy chain terminator method to analyze DNA fragments which had been isolated on a radiogram. The result revealed that the complementary chain DNA contained a base sequence consisting of 2,056 base pairs as shown in SEQ ID NO:12. An amino acid sequence estimable from the base sequence was as shown in SEQ ID NO:12, and compared with the amino acid sequence containing the N-terminal or the partial amino acid sequence of SEQ ID NO:6, 9 or 10. As a result, it was found that the amino acid sequence of SEQ ID NO:6 corresponds to that located at positions from 2 to 21 in SEQ ID NO:12, and that the partial amino acid sequence in SEQ ID NO:9 or 10 corresponds to that located at positions from 470 to 489 or at positions from 11 to 30 in SEQ ID NO:12. These results indicate that enzyme Q36 has the amino acid sequence of SEQ ID NO:4, and it is encoded by the DNA having the base sequence as shown in SEQ ID NO:3.

#### Example 5

##### Preparation of recombinant enzyme

In 500-ml Erlenmeyer flasks were placed 100 ml aliquots of a liquid nutrient culture medium (pH 7.0) consisting of 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate, and to each flask was added 50 µg/ml ampicillin and autoclaved at 120°C for 20 min. Thereafter, the flasks were cooled and inoculated with a seed culture of the transformant BMU27 obtained in Example 1-2, followed by culturing the transformant at 27°C for 24 hours by a rotary shaker. Apart from this, 18 L of a fresh preparation of the same liquid culture medium was placed in a 30-L jar fermentor, admixed with 50 µg/ml ampicillin, sterilized at 120°C for 20 min, cooled and inoculated with one v/v % of the seed culture obtained in the above, followed by the culture at 37°C for 24 hours while keeping the pH at 6-8 under aeration-agitation conditions. The resultant culture was treated with an ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances. The supernatant thus obtained was assayed for the enzyme activity to reveal that one L of the culture yielded about 3,900 units of the enzyme. The supernatant was purified by the method in Experiment 1-1 to obtain an about 67 ml aqueous solution containing an about 165 units/ml of a recombinant enzyme having a specific activity of about 290 units/mg protein.

#### Example 6

##### Preparation of recombinant enzyme

Recombinant BRT32 obtained by the method in Experiment 3-2 was cultured similarly as in Example 5, and the resultant culture was treated with an ultrasonic integrator to disrupt cells. The resultant suspension was centrifuged to remove insoluble substances, and the resultant supernatant was assayed for the enzyme activity to have an activity of about 4,000 units per L. The supernatant was purified by the method in Experiment 1-1 to obtain an about 55 ml aqueous solution containing about 200 units/ml of a recombinant enzyme with a specific activity of about 420 units/mg protein.

#### Example 7

##### Conversion of non-reducing saccharide by recombinant enzyme

##### Example 7-1 (a)

##### Preparation of non-reducing saccharide-forming enzyme

To 500-ml Erlenmeyer flasks were placed 100 ml aliquots of a liquid nutrient culture medium (pH 7.0) consisting of 2.0 w/v % maltose, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate, and the flasks were autoclaved at 120°C for 20 min. Thereafter, the flasks were cooled and inoculated with a seed culture of *Rhizobium* sp. M-11, followed by culturing it at 27°C for 24 hours by a rotary shaker. Apart from this, 20 L of a fresh preparation of the same liquid culture medium was placed in a 30-L jar fermentor, and sterilized, inoculated with one v/v % of the seed culture obtained in the above, followed by the culture at 30°C and at a pH of 7-8 for 24 hours under aeration-agitation conditions. Thereafter, the resultant culture was treated with an ultrasonic disintegrator to disrupt cells, and



the resultant suspension was centrifuged to remove insoluble substances and purified according to the method in Experiment 1-1 to obtain a non-reducing saccharide-forming enzyme having a specific activity of about 195 units/mg protein in a yield of about 220 units per L of the culture.

Throughout the specification the activity of a non-reducing saccharide-forming enzyme is expressed by the value measured on the following assay: Place 4 ml of 50 mM phosphate buffer (pH 7.0) containing 1.25 w/v % maltopentaose in a test tube, add one ml of an enzyme solution to the test tube, and incubate the solution at 40°C for 60 min to effect enzymatic reaction. Thereafter, the reaction mixture is heated at 100°C for 10 min to suspend the enzymatic reaction, followed by diluting it with distilled water by 10 times and assaying the reducing activity by the Somogyi-Nelson's method. One unit activity of the non-reducing saccharide-forming enzyme is defined as the amount of enzyme which decreases the reducing power corresponding to one  $\mu$ mol maltopentaose per min under the above conditions.

#### Example 7-1(b)

##### Preparation of syrupy product containing trehalose

A potato starch was suspended in water to give a 15 w/w % suspension which was then mixed with 0.1 w/w % calcium carbonate. The mixture was adjusted its pH to 6.0, mixed with 0.2 w/w %, d.s.b., of "TERMAMYL 60L", an  $\alpha$ -amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, and enzymatically reacted at 95°C for 15 min to effect gelatinization and liquefaction. The liquefied solution was autoclaved at 120°C for 30 min to inactivate the remaining enzyme, rapidly cooled to 45°C, 1,000 units/g starch, d.s.b., of pullulanase commercialized by Hayashibara Biochemical Laboratories., Inc., Okayama, Japan, 3.4 units/g starch, d.s.b., of the non-reducing saccharide-forming enzyme obtained in Example 7-1(a), and 4.2 units/g starch, d.s.b., of the recombinant enzyme obtained by the method in Example 5, followed the enzymatic reaction for 48 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion-exchange resin, and concentrated to obtain a syrupy product with a concentration of about 60 w/w % in a yield of about 92%, d.s.b.

Analysis of the syrup by the method of Experiment 2-1 revealed that it contained 70.2 w/w % trehalose, 2.4 w/w %  $\alpha$ -glucosyltrehalose, 3.3 w/w %  $\alpha$ -maltosyltrehalose, 0.7 w/w % glucose, 10.1 w/w % maltose, 12.9 w/w % maltotriose, and 0.4 w/w % maltooligosaccharides having a degree of glucose polymerization of 4 or higher. The product, having a mild and moderate sweetness as well as an adequate viscosity and moisture-retaining ability, can be satisfactorily used in food products in general, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

#### Example 7-1(c)

##### Preparation of powdery product containing trehalose

To 4 jacketed-stainless steel columns, having a diameter of 5.4 cm and a length of 5 m each was packed homogeneity with "XT-1016 (Na<sup>+</sup>-form)", a strong-acid cation exchange resin commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, and the columns were cascaded in series to give a total column length of 20 m. The syrupy product obtained in Example 7-1(b) was fed to the columns at a rate of about 5 v/v % against the resin at an inner column temperature of 55°C, and the columns were fed with 55°C hot water at an SV (space velocity) 0.3 to fractionate saccharides in the syrupy product. Based on the analysis of the saccharide composition of the eluate, fractions rich in trehalose were collected, pooled, concentrated, dried *in vacuo* and pulverized to obtain a solid product containing about 97 w/w % trehalose in a yield of about 56% against the starting material, d.s.b.

The product, having a mild sweetness and substantially free of reducibility, can be satisfactorily used in food products in general, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

#### Example 7-1(d)

##### Preparation of powdery crystalline trehalose

A portion of the trehalose rich fraction obtained in Example 7-1(c) was concentrated into an about 75 w/w % solution which was then transferred to a crystallizer, admixed with about 2 w/w %, d.s.b., hydrous crystalline

trehalose as a seed crystal, and crystallized under gentle stirring conditions to obtain a masseccuite with a crystallinity of about 45 w/w %. The masseccuite was sprayed downward from a nozzle, equipped at the upper part of a spraying tower at a pressure of about 150 kg/cm<sup>2</sup> while about 85°C hot air was flowing downward from the upper part of the tower to accumulate a crystalline powder on a belt conveyer provided on the basement of the tower, followed by gradually transferring it out of the tower. Thereafter, the powder was transferred to an ageing tower and aged for 10 hours to complete the crystallization and drying while an about 40°C hot air was blowing to the contents. Thus, a powdery product containing hydrous crystalline trehalose was obtained in a yield of about 90 w/w % against the starting material, d.s.b.

The product, having a substantial non-hygroscopicity and a mild and high-quality sweetness, can be satisfactorily used in food products in general, cosmetics, pharmaceuticals and feeds as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

#### Example 8

##### Conversion of non-reducing saccharide by recombinant enzyme

Potato starch was suspended in water to give a concentration of 6 w/w %, d.s.b., and the suspension was admixed with 500 units/g starch of isoamylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and enzymatically reacted for 20 hours. The reaction mixture was adjusted to a pH of 6.5, autoclaved at 120°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 95°C, admixed with 0.1 w/w % per g starch, d.s.b., of "TERMAMYL 60L", an  $\alpha$ -amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, and enzymatically reacted for 15 min. The reaction mixture was heated at 130°C for 30 min to inactivate the remaining enzyme, rapidly cooled to 45°C, admixed with 4.1 units/g starch, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 7-1(a), and 4.9 units/g starch, d.s.b., of the present recombinant enzyme obtained by the method in Example 6, and enzymatically reacted for 64 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 55°C, adjusted to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and enzymatically reacted for 40 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion-exchange resin, and concentrated to obtain an about 60 w/w % syrupy product containing about 80.5 w/w % trehalose, d.s.b. The syrupy product was concentrated into an about 84 w/w % syrup which was then transferred to a crystallizer, admixed with an about 2 w/w % hydrous crystalline trehalose, d.s.b., and crystallized under gentle stirring conditions to obtain a masseccuite having a crystallinity of about 45 w/w %. The masseccuite was distributed to plastic plain vessels which were then allowed to stand at ambient temperature for 3 days to effect solidification and aging, followed by detaching the resultant blocks from the vessels and pulverizing the blocks with a cutter to obtain a solid product containing hydrous crystalline trehalose in a yield of about 90 w/w % against the material starch, d.s.b.

The product, which is substantially free of hygroscopicity and readily handleable, can be arbitrarily used in food products in general, cosmetics, pharmaceuticals as a sweetening agent, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

#### Example 9

##### Conversion of non-reducing saccharide by recombinant enzyme

Potato starch was suspended in water to give a concentration of 6 w/w %, d.s.b., and the suspension was admixed with 0.01 w/w % "NEO-SPITASE",  $\alpha$ -amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, adjusted to pH 6.2, and enzymatically reacted at 85-90°C for 20 min to gelatinize and liquefy the starch. The liquefied starch was heated at 120°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 45°C, admixed with 500 units/g starch, d.s.b., of isoamylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 3.2 units/g starch, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 7-1(a), and 5.0 units/g starch, d.s.b., of the present recombinant enzyme obtained by the method in Example 5, and enzymatically reacted for 48 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 55°C, adjusted to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals Ltd., Kyoto, Japan, and enzymatically reacted for 40 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, rapidly cooled, filtered, and, in usual manner, decolored with an activated charcoal, de-

salted and purified with an ion-exchange resin, and concentrated to give a concentration of about 60 w/w %, d.s.b., to obtain a syrupy product containing 78.3 w/w % trehalose, d.s.b. The syrupy product was fractionated similarly as in Example 7-1(c) except for using "CG6000(Na<sup>+</sup>)", a strong-acid cation exchange resin commercialized by Japan Organo, Co., Ltd., Tokyo, Japan, to obtain a fraction containing about 95 w/w % trehalose, d.s.b. The fraction was concentrated to give a concentration of about 75 w/w %, d.s.b., and, similarly as in Example 8, crystallized, and the resultant masseccuite in the form of block was pulverized to obtain a powdery product containing hydrous crystalline trehalose in a yield of about 70 w/w % against the material starch, d.s.b.

The product, which is substantially free of hygroscopicity and readily handleable, can be arbitrarily used in food products in general, cosmetics, pharmaceuticals as a sweetening agent, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

As is described above, the present invention is based on the finding that a novel enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. The present invention is to explore a way to produce the enzyme in a relatively-large scale and in a considerably-high yield. The enzyme produced by the transformant according to the present invention is the one characterized by its revealed total amino acid sequence, and because of this it can be used for the preparations of trehalose which is premised on being used in food products without fear of causing side effects.

Therefore, the present invention is an useful invention which exerts the aforesaid significant action and effect as well as giving a great contribution to this field.

## SEQUENCE LISTING

5

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

10

NAME:KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU  
KENKYUJO

(ii) TITLE OF INVENTION:DNA ENCODING ENZYME, RECOMBINANT DNA  
AND ENZYME, TRANSFORMANT, AND THEIR  
PREPARATIONS AND USES

15

(iii) NUMBER OF SEQUENCES:20

## (iv) ADDRESS:

20

(A) ADDRESSEE:KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU  
KAGAKU KENKYUJO  
(B) STREET:2-3, 1-CHOME, SHIMOISHII  
(C) CITY:OKAYAMA  
(E) COUNTRY:JAPAN  
(F) POSTAL CODE (ZIP):700

## (v) COMPUTER READABLE FORM:

25

(A) MEDIUM TYPE:Floppy disk  
(B) COMPUTER:IBM PC compatible  
(C) OPERATING SYSTEM:PC-DOS/MS-DOS

## (vii) PRIOR APPLICATION DATA:

30

(A1) APPLICATION NUMBER:JP 59840/94  
(B1) FILING DATE:March 7, 1994  
(A2) APPLICATION NUMBER:JP 59834/94  
(B2) FILING DATE:March 7, 1994

## (2) INFORMATION FOR SEQ ID NO:1:

35

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:1767 base pairs  
(B) TYPE:nucleic acid  
(D) TOPOLOGY:linear

## (xi) SEQUENCE DESCRIPTION:SEQ ID NO:1:

40

GCCAAGCCGG TGCAGGGAGC GGGGCGCTTC GATATCTGGG CGCCCGAGGC AGGCACCGTA 60

ACGCTGCTGG CCGGCGGGGA GCGCTACGAG ATGGGCCGCC GCCCGGCAA CGGGCCGGCG 120

GACGAAGGCT GGTGGACGGC CGCGGATGCA CCGACAGGCG CGGACGTGGA CTACGGATAC 180

45

CTGCTCGACG GCGACGAAAT CCCGCTGCCG GACCCCGGA CCCGCCCA GCCCGAAGGC 240

GTCCATGCCC TGTCCCGGAC CTTCGACCCC GCGCGCCACC GCTGGCAGGA CGCCGGGTGG 300

CAGGGCAGGG AACTCCAGGG CTCCGTGATT TACGAACTCC ACATCGGAAC GTTCACGCCG 360

50

GAAGGGACGC TGGACGCCGC CGCGGGCAAG CTGGACTACC TCGCCGGCCT GGGCATCGAC 420

TTCATTGAGC TGCTGCCCGT GAATGCCTTC AACGGCACGC ACAACTGGGG CTACGACGGC 480

GTCCAGTGGT TTGCCGTGCA TGAAGGCTAC GCGGGGCCTG CGGCGTACCA GCGGTTCGTG 540

55

GATGCGGCCC ACGCGGCCGG CCTCGGCGTC ATCCAGGACG TGGTCTACAA CCACCTCGGG 600

5 CCGAGCGGGA ACTACCTCCC CAGGTACGGC CCGTACCTCA AGCACGGCGA AGGCAACACC 660  
 TGGGGCGATT CGGTCAACCT GGACGGGCGG GGATCCGACC ACGTCCGCCA GTACATCCTG 720  
 GACAACGTGG CCATGTGGCT GCGCGACTAC CGGGTGGACG GCCTCCGCCT GGACGCCGTC 780  
 10 CACGCCCTGA AGGATGAGCG GGCCGTCCAC ATCCTGGAGG AGTTCGGCGC GCTGGCGGAC 840  
 GCCCTGTCGT CCGAAGGCGG CCGCCCCTG ACCCTCATCG CCGAGTCCGA CCTCAACAAT 900  
 CCGCGGCTGC TGTACCCCCG GGATGTCAAC GGCTACGGAC TGGCCGGCCA GTGGAGCGAC 960  
 GACTTCCACC ACGCCGTGCA CGTCAACGTC AGCGGGGAAA CCACCGGCTA CTACAGCGAC 1020  
 15 TTCGACTCGC TCGGAGCCCT CGCCAAGGTC CTGCGTGACG GGTTCCTTCCA CGACGGCAGC 1080  
 TACTCCAGCT TCCGCGGCCG CTGCCACGGC CGGCCGATCA ACTTCAGCGC CGTGCATCCG 1140  
 GCCGCGCTGG TGGTCTGCTC ACAGAACCAT GACCAGATCG GCAACCGGGC CACCGGGGAC 1200  
 20 CGGCTGTCCC AGTCACTTCC GTACGGCAGC CTGGCCCTGG CCGCCGTGCT GACCCTCACC 1260  
 GGTCCGTTCA CGCCCATGCT GTTCATGGGA GAGGAATACG GGGCCACCAC CCCGTGGCAG 1320  
 TTCTTCACCT CGCACCTGA ACCCGAGCTG GGCAAGGCCA CGGCCGAGGG CAGGATCAGG 1380  
 25 GAGTTCGAGC GCATGGGGTG GGATCCCGCC GTCGTGCCCC ATCCGCAGGA TCCGGAGACC 1440  
 TTCACCCGCT CCAAACCTGA CTGGGCGGAA GCGTCCGCCG GCGATCATGC CCGCCTCCTG 1500  
 GAGCTGTACC GCTCGCTTAT CACGCTGCGG CGGTCAACTC CGGAGCTCGC GCGCCTGGGC 1560  
 30 TTTGCGGACA CCGCCGTCGA GTTCGACGAC GACGCCCGCT GGCTCCGTTA TTGGCGCGGA 1620  
 GGCGTGCAGG TGGTGCTGAA CTTGCGGGAC CGTCCCATCA GCCTGGACCG GCCGGGAACC 1680  
 GCGCTGCTGC TCGCCACCGA CGACGCCGTC CGGATGGACG GAGTCCAGGT GGAGCTGCCG 1740  
 35 CCGCTGAGCG CCGCGGTTCT GCGCGAC 1767

## (3) INFORMATION FOR SEQ ID NO:2:

- 40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 589  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

45 Ala Lys Pro Val Gln Gly Ala Gly Arg Phe Asp Ile Trp Ala Pro Glu Ala  
 1 5 10 15  
 Gly Thr Val Thr Leu Leu Ala Gly Gly Glu Arg Tyr Glu Met Gly Arg Arg  
 20 25 30  
 Pro Gly Asn Gly Pro Ala Asp Glu Gly Trp Trp Thr Ala Ala Asp Ala Pro  
 35 40 45 50  
 50 Thr Gly Ala Asp Val Asp Tyr Gly Tyr Leu Leu Asp Gly Asp Glu Ile Pro  
 55 60 65  
 Leu Pro Asp Pro Arg Thr Arg Arg Gln Pro Glu Gly Val His Ala Leu Ser  
 70 75 80 85  
 Arg Thr Phe Asp Pro Gly Ala His Arg Trp Gln Asp Ala Gly Trp Gln Gly  
 90 95 100

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Arg | Glu | Leu | Gln | Gly | Ser | Val | Ile | Tyr | Glu | Leu | His | Ile | Gly | Thr | Phe | Thr |
|    |     |     | 105 |     |     |     |     | 110 |     |     |     |     | 115 |     |     |     |     |
| 5  | Pro | Glu | Gly | Thr | Leu | Asp | Ala | Ala | Ala | Gly | Lys | Leu | Asp | Tyr | Leu | Ala | Gly |
|    | 120 |     |     |     |     | 125 |     |     |     |     | 130 |     |     |     |     | 135 |     |
|    | Leu | Gly | Ile | Asp | Phe | Ile | Glu | Leu | Leu | Pro | Val | Asn | Ala | Phe | Asn | Gly | Thr |
|    |     |     |     | 140 |     |     |     |     | 145 |     |     |     |     | 150 |     |     |     |
|    | His | Asn | Trp | Gly | Tyr | Asp | Gly | Val | Gln | Trp | Phe | Ala | Val | His | Glu | Gly | Tyr |
|    | 155 |     |     |     |     | 160 |     |     |     |     |     | 165 |     |     |     |     | 170 |
| 10 | Gly | Gly | Pro | Ala | Ala | Tyr | Gln | Arg | Phe | Val | Asp | Ala | Ala | His | Ala | Ala | Gly |
|    |     |     |     | 175 |     |     |     |     |     | 180 |     |     |     |     | 185 |     |     |
|    | Leu | Gly | Val | Ile | Gln | Asp | Val | Val | Tyr | Asn | His | Leu | Gly | Pro | Ser | Gly | Asn |
|    | 190 |     |     |     |     |     |     | 195 |     |     |     |     | 200 |     |     |     |     |
|    | Tyr | Leu | Pro | Arg | Tyr | Gly | Pro | Tyr | Leu | Lys | His | Gly | Glu | Gly | Asn | Thr | Trp |
|    | 205 |     |     |     | 210 |     |     |     |     |     | 215 |     |     |     |     | 220 |     |
| 15 | Gly | Asp | Ser | Val | Asn | Leu | Asp | Gly | Pro | Gly | Ser | Asp | His | Val | Arg | Gln | Tyr |
|    |     |     |     | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |
|    | Ile | Leu | Asp | Asn | Val | Ala | Met | Trp | Leu | Arg | Asp | Tyr | Arg | Val | Asp | Gly | Leu |
|    | 240 |     |     |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |
|    | Arg | Leu | Asp | Ala | Val | His | Ala | Leu | Lys | Asp | Glu | Arg | Ala | Val | His | Ile | Leu |
|    |     |     |     | 260 |     |     |     |     |     | 265 |     |     |     |     | 270 |     |     |
| 20 | Glu | Glu | Phe | Gly | Ala | Leu | Ala | Asp | Ala | Leu | Ser | Ser | Glu | Gly | Gly | Arg | Pro |
|    |     |     | 275 |     |     |     |     | 280 |     |     |     |     |     | 285 |     |     |     |
|    | Leu | Thr | Leu | Ile | Ala | Glu | Ser | Asp | Leu | Asn | Asn | Pro | Arg | Leu | Leu | Tyr | Pro |
|    | 290 |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     | 305 |     |
|    | Arg | Asp | Val | Asn | Gly | Tyr | Gly | Leu | Ala | Gly | Gln | Trp | Ser | Asp | Asp | Phe | His |
|    |     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |     |     |     |
| 25 | His | Ala | Val | His | Val | Asn | Val | Ser | Gly | Glu | Thr | Thr | Gly | Tyr | Tyr | Ser | Asp |
|    | 325 |     |     |     |     | 330 |     |     |     |     |     | 335 |     |     |     |     | 340 |
|    | Phe | Asp | Ser | Leu | Gly | Ala | Leu | Ala | Lys | Val | Leu | Arg | Asp | Gly | Phe | Phe | His |
|    |     |     |     | 345 |     |     |     |     | 350 |     |     |     |     | 355 |     |     |     |
|    | Asp | Gly | Ser | Tyr | Ser | Ser | Phe | Arg | Gly | Arg | Cys | His | Gly | Arg | Pro | Ile | Asn |
|    |     |     |     | 360 |     |     |     | 365 |     |     |     |     | 370 |     |     |     |     |
| 30 | Phe | Ser | Ala | Val | His | Pro | Ala | Ala | Leu | Val | Val | Cys | Ser | Gln | Asn | His | Asp |
|    | 375 |     |     |     |     | 380 |     |     |     |     | 385 |     |     |     |     | 390 |     |
|    | Gln | Ile | Gly | Asn | Arg | Ala | Thr | Gly | Asp | Arg | Leu | Ser | Gln | Ser | Leu | Pro | Tyr |
|    |     |     |     | 395 |     |     |     |     | 400 |     |     |     |     | 405 |     |     |     |
|    | Gly | Ser | Leu | Ala | Leu | Ala | Ala | Val | Leu | Thr | Leu | Thr | Gly | Pro | Phe | Thr | Pro |
|    | 410 |     |     |     |     | 415 |     |     |     |     |     | 420 |     |     |     |     | 425 |
| 35 | Met | Leu | Phe | Met | Gly | Glu | Glu | Tyr | Gly | Ala | Thr | Thr | Pro | Trp | Gln | Phe | Phe |
|    |     |     |     | 430 |     |     |     |     |     | 435 |     |     |     |     | 440 |     |     |
|    | Thr | Ser | His | Pro | Glu | Pro | Glu | Leu | Gly | Lys | Ala | Thr | Ala | Glu | Gly | Arg | Ile |
|    |     |     | 445 |     |     |     |     | 450 |     |     |     |     | 455 |     |     |     |     |
|    | Arg | Glu | Phe | Glu | Arg | Met | Gly | Trp | Asp | Pro | Ala | Val | Val | Pro | Asp | Pro | Gln |
|    | 460 |     |     |     |     | 465 |     |     |     |     | 470 |     |     |     | 475 |     |     |
| 40 | Asp | Pro | Glu | Thr | Phe | Thr | Arg | Ser | Lys | Leu | Asp | Trp | Ala | Glu | Ala | Ser | Ala |
|    |     |     |     | 480 |     |     |     |     | 485 |     |     |     |     | 490 |     |     |     |
|    | Gly | Asp | His | Ala | Arg | Leu | Leu | Glu | Leu | Tyr | Arg | Ser | Leu | Ile | Thr | Leu | Arg |
|    | 495 |     |     |     |     | 500 |     |     |     |     |     | 505 |     |     |     |     | 510 |
|    | Arg | Ser | Thr | Pro | Glu | Leu | Ala | Arg | Leu | Gly | Phe | Ala | Asp | Thr | Ala | Val | Glu |
|    |     |     |     | 515 |     |     |     |     |     | 520 |     |     |     |     | 525 |     |     |
| 45 | Phe | Asp | Asp | Asp | Ala | Arg | Trp | Leu | Arg | Tyr | Trp | Arg | Gly | Gly | Val | Gln | Val |
|    |     |     | 530 |     |     |     |     | 535 |     |     |     |     | 540 |     |     |     |     |
|    | Val | Leu | Asn | Phe | Ala | Asp | Arg | Pro | Ile | Ser | Leu | Asp | Arg | Pro | Gly | Thr | Ala |
|    | 545 |     |     |     |     | 550 |     |     |     |     | 555 |     |     |     |     | 560 |     |
|    | Leu | Leu | Leu | Ala | Thr | Asp | Asp | Ala | Val | Arg | Met | Asp | Gly | Val | Gln | Val | Glu |
|    |     |     |     | 565 |     |     |     |     | 570 |     |     |     |     | 575 |     |     |     |
| 50 | Leu | Pro | Pro | Leu | Ser | Ala | Ala | Val | Leu | Arg | Asp |     |     |     |     |     |     |
|    |     |     |     | 580 |     |     |     | 585 |     |     |     |     |     |     |     |     |     |

55 (4) INFORMATION FOR SEQ ID NO:3:  
(i) SEQUENCE CHARACTERISTICS:

EP 0 671 470 A2

5 (A) LENGTH: 1791 base pairs  
(B) TYPE: nucleic acid  
(D) TOPOLOGY: linear  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

|    |   |      |
|----|---|------|
| 10 | ACGCACACCT ACCCGCGGGA AGCCGCGAAA CCCGTCCTGG GCCCCGCACG CTACGACGTC | 60   |
|    | TGGGCGCCCA ACGCTGAATC CGTGACGCTG CTGGCCGGCG GGGAGCGCTA CGCCATGCAG | 120  |
|    | CGCCGGGCGG AGACCGGGCC GGAGGACGCC GGCTGGTGGA CCGCCGCCGG CGCGCCTACG | 180  |
| 15 | GATGGCAACG TGGACTACGG GTACCTTCTG GACGGCGACG AAACACCGCT TCCGGATCCA | 240  |
|    | CGGACCCGCC GCCAGCCCGA CGGCGTCCAC GCCCTGTCCC GCACGTTCGA CCCGTCCGCG | 300  |
|    | TACAGCTGGC AGGACGACGC CTGGCAGGGC AGGGAAGTGC AGGGCGCCGT CATCTACGAG | 360  |
| 20 | CTCCACCTCG GAACATTAC GCCCGAAGGG ACGCTGGAGG CGGCCGCCGG AAAGCTGGAC  | 420  |
|    | TACCTCGCCG GCTTGGGCGT CGACTTCATC GAGCTGCTGC CGGTGAACGC TTTCAACGGC | 480  |
|    | ACGCACAAC TGGGTTACGA CGGTGTCCAG TGCTTCGCTG TGCACGAGGC ATACGGCGGG  | 540  |
| 25 | CCGGAAGCGT ACCAGCGGTT CGTCGACGCC GCCCAGCCG CAGGCCCTTG CGTGATCCAG  | 600  |
|    | GACGTGGTCT ACAACCACCT CGGCCCCAGC GGGAAGTACC TGCCGCGGTT CGGGCCGTAC | 660  |
|    | CTCAAGCAGG GCGAGGGTAA CACGTGGGGC GACTCGGTGA ACCTGGACGG GCCCGGCTCC | 720  |
| 30 | GACCATGTGC GCCGGTACAT CCTGGACAAC CTGGCCATGT GGCTGCGTGA CTACCGGGTG | 780  |
|    | GACGGCCTGC GGCTGGACGC CGTCCACGCC CTGAAGGATG AGCGGGCGGT GCACATCCTG | 840  |
|    | GAGGACTTCG GGGCGCTGGC CGATCAGATC TCCGCCGAGG TGGGACGGCC GCTGACGCTC | 900  |
| 35 | ATCGCCGAGT CCGACCTCAA CAACCCCGCG CTGCTGTACC CGCGGGACGT CAACGGGTAC | 960  |
|    | GGGCTGGAAG GGCAGTGGAG CGACGACTTC CACCACGCCG TCCACGTCAA CGTCACCGGC | 1020 |
|    | GAAACCACCG GCTACTACAG TGAATTCGAC TCGCTGGCCG CCCTCGCCAA GGTGCTCCGG | 1080 |
| 40 | GACGGCTTCT TCCACGACGG CAGTACTTCC AGCTTCCGGG AACGCCACCA CGGACGGCCG | 1140 |
|    | ATTAATTTCA GCGCCGTACA CCCAGCCGCC CTGGTGGTCT GTTCGCAGAA CCACGACCAG | 1200 |
|    | ATCGGCAACC GTGCCACGGG GGACCGGCTC TCCAGACCC TGCCGTACGG AAGCCTGGCC  | 1260 |
|    | CTCGCTGCGG TGCTGACCCT GACGGGACCC TTCACGCCA TGCTGCTCAT GGGCGAGGAG  | 1320 |
| 45 | TACGGCGCCA GCACGCCGTG GCAGTTTTC ACCTCGCACC CGGAGCCGGA GCTCGGCAAG  | 1380 |
|    | GCCACCGCGG AGGGCCGGAT CAAGGAGTTC GAGCGCATGG GGTGGGATCC CGCCGTCTGT | 1440 |
|    | CCCAGTCCCC AGGATCCTGA GACGTTCCGC CGGTCCAAGC TGGACTGGGC GGAAGCCGCC | 1500 |
| 50 | GAAGGCGACC ATGCCCGGCT GCTGGAGCTG TACCGTTCGC TCACCGCCCT GCGCCGCTCC | 1560 |
|    | ACGCCGACG TCACCAAGCT GGGCTTCGAG GACACGACG TGGCGTTCGA CGAGGACGCC   | 1620 |
|    | CGCTGGCTGC GGTTCGCGG GGGTGGCGTG CAGGTGCTGC TCAACTTCTC GGAACAGCCC  | 1680 |
| 55 | GTGAGCCTGG ACGGGGCGGG CACGGCCCTG CTGCTGGCCA CCGACGACGC CGTCCGGCTA | 1740 |

GAAGGTGAGC GTGCGGAAC TCGTCCGCTG AGCGCCGCCG TCGTCAGCGA C

1791

## (5) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:597

(B) TYPE:amino acid

(D) TOPOLOGY:linear

## (ii) MOLECULE TYPE:peptide

## (xi) SEQUENCE DESCRIPTION:SEQ ID NO:4:

15 Thr His Thr Tyr Pro Arg Glu Ala Ala Lys Pro Val Leu Gly Pro Ala Arg  
 1 5 10 15  
 Tyr Asp Val Trp Ala Pro Asn Ala Glu Ser Val Thr Leu Ala Gly Gly  
 20 20 25 30  
 Glu Arg Tyr Ala Met Gln Arg Arg Ala Glu Thr Gly Pro Glu Asp Ala Gly  
 35 40 45 50  
 20 Trp Trp Thr Ala Gly Ala Pro Thr Asp Gly Asn Val Asp Tyr Gly Tyr  
 55 60 65  
 Leu Leu Asp Gly Asp Glu Thr Pro Leu Pro Asp Pro Arg Thr Arg Arg Gln  
 70 75 80 85  
 Pro Asp Gly Val His Ala Leu Ser Arg Thr Phe Asp Pro Ser Ala Tyr Ser  
 90 95 100  
 25 Trp Gln Asp Asp Ala Trp Gln Gly Arg Glu Leu Gln Gly Ala Val Ile Tyr  
 105 110 115  
 Glu Leu His Leu Gly Thr Phe Thr Pro Glu Gly Thr Leu Glu Ala Ala Ala  
 120 125 130 135  
 Gly Lys Leu Asp Tyr Leu Ala Gly Leu Gly Val Asp Phe Ile Glu Leu Leu  
 140 145 150  
 30 Pro Val Asn Ala Phe Asn Gly Thr His Asn Trp Gly Tyr Asp Gly Val Gln  
 155 160 165 170  
 Trp Phe Ala Val His Glu Asp Tyr Gly Gly Pro Glu Ala Tyr Gln Arg Phe  
 175 180 185  
 Val Asp Ala Ala His Ala Ala Gly Leu Gly Val Ile Gln Asp Val Val Tyr  
 190 195 200  
 35 Asn His Leu Gly Pro Ser Gly Asn Tyr Leu Pro Arg Phe Gly Pro Tyr Leu  
 205 210 215 220  
 Lys Gln Gly Glu Gly Asn Thr Trp Gly Asp Ser Val Asn Leu Asp Gly Pro  
 225 230 235  
 Gly Ser Asp His Val Arg Arg Tyr Ile Leu Asp Asn Leu Ala Met Trp Leu  
 240 245 250 255  
 40 Arg Asp Tyr Arg Val Asp Gly Leu Arg Leu Asp Ala Val His Ala Leu Lys  
 260 265 270  
 Asp Glu Arg Ala Val His Ile Leu Glu Asp Phe Gly Ala Leu Ala Asp Gln  
 275 280 285  
 Ile Ser Ala Glu Val Gly Arg Pro Leu Thr Leu Ile Ala Glu Ser Asp Leu  
 290 295 300 305  
 45 Asn Asn Pro Arg Leu Leu Tyr Pro Arg Asp Val Asn Gly Tyr Gly Leu Glu  
 310 315 320  
 Gly Gln Trp Ser Asp Asp Phe His His Ala Val His Val Asn Val Thr Gly  
 325 330 335 340  
 Glu Thr Thr Gly Tyr Tyr Ser Asp Phe Asp Ser Leu Ala Ala Leu Ala Lys  
 345 350 355  
 50 Val Leu Arg Asp Gly Phe Phe His Asp Gly Ser Tyr Ser Ser Phe Arg Glu  
 360 365 370  
 Arg His His Gly Arg Pro Ile Asn Phe Ser Ala Val His Pro Ala Ala Leu  
 375 380 385 390  
 Val Val Cys Ser Gln Asn His Asp Gln Ile Gly Asn Arg Ala Thr Gly Asp  
 395 400 405  
 55 Arg Leu Ser Gln Thr Leu Pro Tyr Gly Ser Leu Ala Leu Ala Val Leu  
 410 415 420 425



5 Thr Leu Thr Gly Pro Phe Thr Pro Met Leu Leu Met Gly Glu Glu Tyr Gly  
 Ala Ser Thr Pro Trp Gln Phe Phe Thr Ser His Pro Glu Pro Glu Leu Gly  
 Lys Ala Thr Ala Glu Gly Arg Ile Lys Glu Phe Glu Arg Met Gly Trp Asp  
 460 445 450 455 465 470 475  
 Pro Ala Val Val Pro Asp Pro Gln Asp Pro Glu Thr Phe Arg Arg Ser Lys  
 480 485 490  
 10 Leu Asp Trp Ala Glu Ala Ala Glu Gly Asp His Ala Arg Leu Leu Glu Leu  
 495 500 505 510  
 Tyr Arg Ser Leu Thr Ala Leu Arg Arg Ser Thr Pro Asp Leu Thr Lys Leu  
 515 520 525  
 Gly Phe Glu Asp Thr Gln Val Ala Phe Asp Glu Asp Ala Arg Trp Leu Arg  
 530 535 540  
 15 Phe Arg Arg Gly Gly Val Gln Val Leu Leu Asn Phe Ser Glu Gln Pro Val  
 545 550 555 560  
 Ser Leu Asp Gly Ala Gly Thr Ala Leu Leu Leu Ala Thr Asp Asp Ala Val  
 565 570 575  
 Arg Leu Glu Gly Glu Arg Ala Glu Leu Gly Pro Leu Ser Ala Ala Val Val  
 580 585 590 595  
 20 Ser Asp

## (6) INFORMATION FOR SEQ ID NO:5:

25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH:20  
 (B) TYPE:amino acid  
 (D) TOPOLOGY:linear  
 (ii) MOLECULE TYPE:peptide  
 (v) FRAGMENT TYPE:N-terminal fragment  
 30 (xi) SEQUENCE DESCRIPTION:SEQ ID NO:5:

Ala Lys Pro Val Gln Gly Ala Gly Arg Phe Asp Ile Trp Ala Pro Glu Ala  
 1 5 10 15  
 Gly Thr Val  
 20  
 35

## (7) INFORMATION FOR SEQ ID NO:6:

40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH:20  
 (B) TYPE:amino acid  
 (D) TOPOLOGY:linear  
 (ii) MOLECULE TYPE:peptide  
 (v) FRAGMENT TYPE:N-terminal fragment  
 45 (xi) SEQUENCE DESCRIPTION:SEQ ID NO:6:

Thr His Thr Tyr Pro Arg Glu Ala Ala Lys Pro Val Leu Gly Pro Ala Arg  
 1 5 10 15  
 Tyr Asp Val  
 20  
 50

## (8) INFORMATION FOR SEQ ID NO:7:

55 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH:21  
 (B) TYPE:amino acid  
 (D) TOPOLOGY:linear

5 (ii)MOLECULE TYPE:peptide  
 (v)FRAGMENT TYPE:internal fragment  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:9:

Pro Val Gln Gly Ala Gly Arg Phe Asp Ile Trp Ala Pro Glu Ala Gly Thr  
 1 5 10 15  
 10 Val Thr Leu Leu  
 20

(9)INFORMATION FOR SEQ ID NO:8:  
 15 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:17  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 (v)FRAGMENT TYPE:internal fragment  
 20 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:8:

Leu Asp Trp Ala Glu Ala Ser Ala Gly Asp His Ala Arg Leu Leu Glu Leu  
 1 5 10 15  
 25

(10)INFORMATION FOR SEQ ID NO:9:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:20  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 30 (ii)MOLECULE TYPE:peptide  
 (v)FRAGMENT TYPE:internal fragment  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:9:

Glu Phe Glu Arg Met Gly Trp Asp Pro Ala Val Val Pro Asp Pro Gln Asp  
 35 1 5 10 15  
 Pro Glu Thr  
 20

40 (11)INFORMATION FOR SEQ ID NO:10:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:20  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 45 (v)FRAGMENT TYPE:internal fragment  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:10:

Pro Val Leu Gly Pro Ala Arg Tyr Asp Val Trp Ala Pro Asn Ala Glu Ser  
 1 5 10 15  
 50 Val Thr Leu  
 20

55 (12)INFORMATION FOR SEQ ID NO:11:  
 (i)SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 2161 base pairs  
 (B) TYPE: nucleic acid  
 (C) strandedness: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: genomic DNA  
 (vi) ORIGINAL SOURCE:  
 10 (A) ORGANISM: *Rhizobium* sp.  
 (B) INDIVIDUAL ISOLATE: M-11 (FERM BP-4130)  
 (ix) FEATURE:  
 (A) NAME/KEY: 5'UTR  
 (B) LOCATION: 1..206  
 (C) IDENTIFICATION METHOD: E  
 15 (A) NAME/KEY: mat peptide  
 (B) LOCATION: 207..1994  
 (C) IDENTIFICATION METHOD: S  
 (A) NAME/KEY: 3'UTR  
 (B) LOCATION: 1995..2161  
 (C) IDENTIFICATION METHOD: E  
 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGCGCGGGG GAGTGCTGGC GCTTGCCACC CGGCTCCCCT ACGGGCTGGA ACAGTCGGGC 60  
 GGCTGGCGGG ACACCGCCGT CGAGCTTGAA GCCGCCATGA CGGACGAACT GACCGGCTCC 120  
 25 ACTTTCGGGC CGGGACCGGC GCGCTGTCA GAAGTCTTCC GGGCCTACCC GGTGGCCTTG 180  
 TTGGTCCCCG CGACAGGAGG CAAGTC 206

ATG ACG CAG CCC AAC GAT GCG GCC AAG CCG GTG CAG GGA GCG GGG CGC 254  
 Met Thr Gln Pro Asn Asp Ala Ala Lys Pro Val Gln Gly Ala Gly Arg  
 30 1 5 10 15  
 TTC GAT ATC TGG GCG CCC GAG GCA GGC ACC GTA ACG CTG CTG GCC GGC 302  
 Phe Asp Ile Trp Ala Pro Glu Ala Gly Thr Val Thr Leu Leu Ala Gly  
 20 25 30  
 GGG GAG CGC TAC GAG ATG GGC CGC CGC CCC GGC AAC GGG CCG GCG GAC 350  
 Gly Glu Arg Tyr Glu Met Gly Arg Arg Pro Gly Asn Gly Pro Ala Asp  
 35 35 40 45  
 GAA GGC TGG TGG ACG GCC GCG GAT GCA CCG ACA GGC GCG GAC GTG GAC 398  
 Glu Gly Trp Trp Thr Ala Ala Asp Ala Pro Thr Gly Ala Asp Val Asp  
 50 55 60  
 TAC GGA TAC CTG CTC GAC GGC GAC GAA ATC CCG CTG CCG GAC CCC CGG 446  
 Tyr Gly Tyr Leu Leu Asp Gly Asp Glu Ile Pro Leu Pro Asp Pro Arg  
 65 70 75 80  
 40 ACC CGC CGC CAG CCC GAA GGC GTC CAT GCC CTG TCC CGG ACC TTC GAC 494  
 Thr Arg Arg Gln Pro Glu Gly Val His Ala Leu Ser Arg Thr Phe Asp  
 85 90 95  
 CCC GGC GCC CAC CGC TGG CAG GAC GCC GGG TGG CAG GGC AGG GAA CTC 542  
 Pro Gly Ala His Arg Trp Gln Asp Ala Gly Trp Gln Gly Arg Glu Leu  
 100 105 110  
 45 CAG GGC TCC GTG ATT TAC GAA CTC CAC ATC GGA ACG TTC ACG CCG GAA 590  
 Gln Gly Ser Val Ile Tyr Glu Leu His Ile Gly Thr Phe Thr Pro Glu  
 115 120 125  
 GGG ACG CTG GAC GCC GCC GCG GGC AAG CTG GAC TAC CTC GCC GGC CTG 638  
 Gly Thr Leu Asp Ala Ala Ala Gly Lys Leu Asp Tyr Leu Ala Gly Leu  
 130 135 140  
 50 GGC ATC GAC TTC ATT GAG CTG CTG CCC GTG AAT GCC TTC AAC GGC ACG 686  
 Gly Ile Asp Phe Ile Glu Leu Leu Pro Val Asn Ala Phe Asn Gly Thr  
 145 150 155 160  
 CAC AAC TGG GGC TAC GAC GGC GTC CAG TGG TTT GCC GTG CAT GAA GGC 734  
 His Asn Trp Gly Tyr Asp Gly Val Gln Trp Phe Ala Val His Glu Gly  
 165 170 175  
 55 TAC GGC GGG CCT GCG GCG TAC CAG CGG TTC GTG GAT GCG GCC CAC GCG 782

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|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| 5  | Tyr | Gly | Gly | Pro | Ala | Ala | Tyr | Gln | Arg | Phe | Val | Asp | Ala | Ala | His | Ala |      |
|    | GCC | GGC | CTC | GGC | GTC | ATC | CAG | GAC | GTG | GTC | TAC | AAC | CAC | CTC | GGG | CCG | 830  |
|    | Ala | Gly | Leu | Gly | Val | Ile | Gln | Asp | Val | Val | Tyr | Asn | His | Leu | Gly | Pro |      |
|    |     |     | 195 |     |     |     |     | 200 |     |     |     |     | 205 |     |     |     |      |
|    | AGC | GGG | AAC | TAC | CTC | CCC | AGG | TAC | GGC | CCG | TAC | CTC | AAG | CAC | GGC | GAA | 878  |
|    | Ser | Gly | Asn | Tyr | Leu | Pro | Arg | Tyr | Gly | Pro | Tyr | Leu | Lys | His | Gly | Glu |      |
| 10 |     | 210 |     |     |     |     | 215 |     |     |     |     | 220 |     |     |     |     |      |
|    | GGC | AAC | ACC | TGG | GGC | GAT | TCG | GTC | AAC | CTG | GAC | GGG | CCG | GGA | TCC | GAC | 926  |
|    | Gly | Asn | Thr | Trp | Gly | Asp | Ser | Val | Asn | Leu | Asp | Gly | Pro | Gly | Ser | Asp |      |
|    | 225 |     |     |     |     | 230 |     |     |     |     | 235 |     |     |     |     | 240 |      |
|    | CAC | GTC | CGC | CAG | TAC | ATC | CTG | GAC | AAC | GTG | GCC | ATG | TGG | CTG | CGC | GAC | 974  |
|    | His | Val | Arg | Gln | Tyr | Ile | Leu | Asp | Asn | Val | Ala | Met | Trp | Leu | Arg | Asp |      |
| 15 |     |     |     |     | 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |      |
|    | TAC | CGG | GTG | GAC | GGC | CTC | CGC | CTG | GAC | GCC | GTC | CAC | GCC | CTG | AAG | GAT | 1022 |
|    | Tyr | Arg | Val | Asp | Gly | Leu | Arg | Leu | Asp | Ala | Val | His | Ala | Leu | Lys | Asp |      |
|    |     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |     |      |
|    | GAG | CGG | GCC | GTC | CAC | ATC | CTG | GAG | GAG | TTC | GGC | GCG | CTG | GCG | GAC | GCC | 1070 |
|    | Glu | Arg | Ala | Val | His | Ile | Leu | Glu | Glu | Phe | Gly | Ala | Leu | Ala | Asp | Ala |      |
|    |     |     |     | 275 |     |     |     |     | 280 |     |     |     | 285 |     |     |     |      |
| 20 | CTG | TCG | TCC | GAA | GGC | GGC | CGC | CCG | CTG | ACC | CTC | ATC | GCC | GAG | TCC | GAC | 1118 |
|    | Leu | Ser | Ser | Glu | Gly | Gly | Arg | Pro | Leu | Thr | Leu | Ile | Ala | Glu | Ser | Asp |      |
|    |     | 290 |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     |      |
|    | CTC | AAC | AAT | CCG | CGG | CTG | CTG | TAC | CCC | CGG | GAT | GTC | AAC | GGC | TAC | GGA | 1166 |
|    | Leu | Asn | Asn | Pro | Arg | Leu | Leu | Tyr | Pro | Arg | Asp | Val | Asn | Gly | Tyr | Gly |      |
|    | 305 |     |     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |      |
| 25 | CTG | GCC | GGC | CAG | TGG | AGC | GAC | GAC | TTC | CAC | CAC | GCC | GTG | CAC | GTC | AAC | 1214 |
|    | Leu | Ala | Gly | Gln | Trp | Ser | Asp | Asp | Phe | His | His | Ala | Val | His | Val | Asn |      |
|    |     |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |      |
|    | GTC | AGC | GGG | GAA | ACC | ACC | GGC | TAC | TAC | AGC | GAC | TTC | GAC | TCG | CTC | GGA | 1262 |
|    | Val | Ser | Gly | Glu | Thr | Thr | Gly | Tyr | Tyr | Ser | Asp | Phe | Asp | Ser | Leu | Gly |      |
|    |     |     |     | 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |     |      |
| 30 | GCC | CTC | GCC | AAG | GTC | CTG | CGT | GAC | GGG | TTC | TTC | CAC | GAC | GGC | AGC | TAC | 1310 |
|    | Ala | Leu | Ala | Lys | Val | Leu | Arg | Asp | Gly | Phe | Phe | His | Asp | Gly | Ser | Tyr |      |
|    |     |     |     | 355 |     |     |     | 360 |     |     |     |     | 365 |     |     |     |      |
|    | TCC | AGC | TTC | CGC | GGC | CGC | TGC | CAC | GGC | CGG | CCG | ATC | AAC | TTC | AGC | GCC | 1358 |
|    | Ser | Ser | Phe | Arg | Gly | Arg | Cys | His | Gly | Arg | Pro | Ile | Asn | Phe | Ser | Ala |      |
|    |     | 370 |     |     |     | 375 |     |     |     |     |     | 380 |     |     |     |     |      |
| 35 | GTG | CAT | CCG | GCC | GCG | CTG | GTG | GTC | TGC | TCA | CAG | AAC | CAT | GAC | CAG | ATC | 1406 |
|    | Val | His | Pro | Ala | Ala | Leu | Val | Val | Cys | Ser | Gln | Asn | His | Asp | Gln | Ile |      |
|    |     | 385 |     |     |     | 390 |     |     |     |     | 395 |     |     |     | 400 |     |      |
|    | GGC | AAC | CGG | GCC | ACC | GGG | GAC | CGG | CTG | TCC | CAG | TCA | CTT | CCG | TAC | GGC | 1454 |
|    | Gly | Asn | Arg | Ala | Thr | Gly | Asp | Arg | Leu | Ser | Gln | Ser | Leu | Pro | Tyr | Gly |      |
|    |     |     |     | 405 |     |     |     |     |     | 410 |     |     |     | 415 |     |     |      |
| 40 | AGC | CTG | GCC | CTG | GCC | GCC | GTG | CTG | ACC | CTC | ACC | GGT | CCG | TTC | ACG | CCC | 1502 |
|    | Ser | Leu | Ala | Leu | Ala | Ala | Val | Leu | Thr | Leu | Thr | Gly | Pro | Phe | Thr | Pro |      |
|    |     |     |     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |      |
|    | ATG | CTG | TTC | ATG | GGA | GAG | GAA | TAC | GGG | GCC | ACC | ACC | CCG | TGG | CAG | TTC | 1550 |
|    | Met | Leu | Phe | Met | Gly | Glu | Glu | Tyr | Gly | Ala | Thr | Thr | Pro | Trp | Gln | Phe |      |
|    |     |     |     | 435 |     |     |     | 440 |     |     |     |     | 445 |     |     |     |      |
| 45 | TTC | ACC | TCG | CAC | CCT | GAA | CCC | GAG | CTG | GGC | AAG | GCC | ACG | GCC | GAG | GGC | 1598 |
|    | Phe | Thr | Ser | His | Pro | Glu | Pro | Glu | Leu | Gly | Lys | Ala | Thr | Ala | Glu | Glu |      |
|    |     | 450 |     |     |     | 455 |     |     |     |     |     | 460 |     |     |     |     |      |
|    | AGG | ATC | AGG | GAG | TTC | GAG | CGC | ATG | GGG | TGG | GAT | CCC | GCC | GTC | GTG | CCC | 1646 |
|    | Arg | Ile | Arg | Glu | Phe | Glu | Arg | Met | Gly | Trp | Asp | Pro | Ala | Val | Val | Pro |      |
|    |     | 465 |     |     |     | 470 |     |     |     |     | 475 |     |     |     | 480 |     |      |
| 50 | GAT | CCG | CAG | GAT | CCG | GAG | ACC | TTC | ACC | CGC | TCC | AAA | CTG | GAC | TGG | GCG | 1694 |
|    | Asp | Pro | Gln | Asp | Pro | Glu | Thr | Phe | Thr | Arg | Ser | Lys | Leu | Asp | Trp | Ala |      |
|    |     |     |     | 485 |     |     |     |     |     | 490 |     |     |     | 495 |     |     |      |
|    | GAA | GCG | TCC | GCC | GGC | GAT | CAT | GCC | CGC | CTC | CTG | GAG | CTG | TAC | CGC | TCG | 1742 |
|    | Glu | Ala | Ser | Ala | Gly | Asp | His | Ala | Arg | Leu | Leu | Glu | Leu | Tyr | Arg | Ser |      |
|    |     |     |     | 500 |     |     |     |     | 505 |     |     |     |     | 510 |     |     |      |
| 55 | CTT | ATC | ACG | CTG | CGG | CGG | TCA | ACT | CCG | GAG | CTC | GCG | CGC | CTG | GGC | TTT | 1790 |

5 Leu Ile Thr Leu Arg Arg Ser Thr Pro Glu Leu Ala Arg Leu Gly Phe  
 515 520 525  
 GCG GAC ACC GCC GTC GAG TTC GAC GAC GAC GCC CGC TGG CTC CGT TAT 1838  
 Ala Asp Thr Ala Val Glu Phe Asp Asp Asp Ala Arg Trp Leu Arg Tyr  
 530 535 540  
 TGG CGC CGA GGC GTG CAG GTG GTG CTG AAC TTC GCG GAC CGT CCC ATC 1886  
 Trp Arg Gly Gly Val Gln Val Val Leu Asn Phe Ala Asp Arg Pro Ile  
 545 550 555 560  
 10 AGC CTG GAC CGG CCG GGA ACC GCG CTG CTG CTC GCC ACC GAC GAC GCC 1934  
 Ser Leu Asp Arg Pro Gly Thr Ala Leu Leu Leu Ala Thr Asp Asp Ala  
 565 570 575  
 GTC CGG ATG GAC GGA GTC CAG GTG GAG CTG CCG CCG CTG AGC GCC GCG 1982  
 Val Arg Met Asp Gly Val Gln Val Glu Leu Pro Pro Leu Ser Ala Ala  
 580 585 590  
 15 GTT CTG CGC GAC 1994  
 Val Leu Arg Asp  
 595  
 TGAGCGTGCG CGCCTTCGGG GCGGGCGTCC TTCCGGTGAC CGGATGCTGG ACGCCCGCCC 2054  
 20 CGCAGCTCCA CAGGCGCTGG CAGGATGGAA CGTATGACTT TTCTGGCAGC GGACAACCGC 2114  
 TACGAAACCA TGCCATACCG CCGCGTCGGA CGCAGCGGGC TGAAGCT 2161

25 (13) INFORMATION FOR SEQ ID NO:12:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2056 base pairs  
 (B) TYPE: nucleic acid  
 (C) strandedness: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: genomic DNA  
 30 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Arthrobacter sp.  
 (B) INDIVIDUAL ISOLATE: Q36 (FERM BP-4316)  
 (ix) FEATURE:  
 (A) NAME/KEY: 5'UTR  
 (B) LOCATION: 1..89  
 35 (C) IDENTIFICATION METHOD: E  
 (A) NAME/KEY: mat peptide  
 (B) LOCATION: 90..1883  
 (C) IDENTIFICATION METHOD: S  
 (A) NAME/KEY: 3'UTR  
 (B) LOCATION: 1884..2056  
 40 (C) IDENTIFICATION METHOD: E  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCGGCTTCG GACCGGGGGC AGTGAAGATC GCCGACATCT TCCGGTCGTT CCCC GTTGCG 60  
 45 CTGCTGGTGC CGCAGACAGG AGGAGAGTC 89  
 ATG ACG CAC ACC TAC CCG CGG GAA GCC GCG AAA CCC GTC CTG GGC CCC 137  
 Met Thr His Thr Tyr Pro Arg Glu Ala Ala Lys Pro Val Leu Gly Pro  
 1 5 10 15  
 50 GCA CGC TAC GAC GTC TGG GCG CCC AAC GCT GAA TCC GTG ACG CTG CTG 185  
 Ala Arg Tyr Asp Val Trp Ala Pro Asn Ala Glu Ser Val Thr Leu Leu  
 20 25 30  
 GCC GGC GGG GAG CGC TAC GCC ATG CAG CGC CGG GCC GAG ACC GGG CCG 233  
 Ala Gly Gly Glu Arg Tyr Ala Met Gln Arg Arg Ala Glu Thr Gly Pro  
 35 40 45  
 GAG GAC GCC GGC TGG TGG ACC GCC GCC GGC GCG CCT ACG GAT GGC AAC 281  
 55 Glu Asp Ala Gly Trp Trp Thr Ala Ala Gly Ala Pro Thr Asp Gly Asn

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|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |      |      |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|
| 5  | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     | 329 |     |      |      |
|    | GTG | GAC | TAC | GGG | TAC | CTT | CTG | GAC | GGC | GAC | GAA | ACA | CCG | CTT | CCG |     | GAT |      |      |
|    | Val | Asp | Tyr | Gly | Tyr | Leu | Leu | Asp | Gly | Asp | Glu | Thr | Pro | Leu | Pro |     | Asp |      |      |
| 10 | 65  | CCA | CGG | ACC | CGC | CGC | CAG | CCC | GAC | GGC | GTC | CAC | GCC | CTG | TCC | CGC | ACG | 377  |      |
|    | Pro | Arg | Thr | Arg | Arg | Gln | Pro | Asp | Gly | Val | His | Ala | Leu | Ser | Arg | Thr |     |      |      |
|    | 85  | TTC | GAC | CCG | TCC | GCG | TAC | AGC | TGG | CAG | GAC | GAC | GCC | TGG | CAG | GGC | AGG |      |      |
| 15 | 100 | Phe | Asp | Pro | Ser | Ala | Tyr | Ser | Trp | Gln | Asp | Asp | Ala | Trp | Gln | Gly | Arg | 425  |      |
|    | 110 | GAA | CTG | CAG | GGC | GCC | GTC | ATC | TAC | GAG | CTC | CAC | CTC | GGA | ACA | TTC | ACG |      |      |
|    | 115 | Glu | Leu | Gln | Gly | Ala | Val | Ile | Tyr | Glu | Leu | His | Leu | Gly | Thr | Phe | Thr |      |      |
| 20 | 120 | CCC | GAA | GGG | ACG | CTG | GAG | GCG | GCC | GCC | GGA | AAG | CTG | GAC | TAC | CTC | GCC | 521  |      |
|    | 130 | Pro | Glu | Gly | Thr | Leu | Glu | Ala | Ala | Ala | Gly | Lys | Leu | Asp | Tyr | Leu | Ala |      |      |
|    | 135 | GGC | TTG | GGC | GTC | GAC | TTC | ATC | GAG | CTG | CTG | CCG | GTG | AAC | GCT | TTC | AAC |      |      |
| 25 | 145 | Gly | Leu | Gly | Val | Asp | Phe | Ile | Glu | Leu | Leu | Pro | Val | Asn | Ala | Phe | Asn | 569  |      |
|    | 150 | GGC | ACG | CAC | AAC | TGG | GGT | TAC | GAC | GGT | GTC | CAG | TGG | TTC | GCT | GTG | CAC |      |      |
|    | 155 | Gly | Thr | His | Asn | Trp | Gly | Tyr | Asp | Gly | Val | Gln | Trp | Phe | Ala | Val | His |      |      |
| 30 | 165 | GAG | GCA | TAC | GGC | GGG | CCG | GAA | GCG | TAC | CAG | CCG | TTC | GTC | GAC | GCC | GCC | 617  |      |
|    | 170 | Glu | Asp | Tyr | Gly | Gly | Pro | Glu | Ala | Tyr | Gln | Arg | Phe | Val | Asp | Ala | Ala |      |      |
|    | 175 | CAC | GCC | GCA | GGC | CTT | GGC | GTG | ATC | CAG | GAC | GTG | GTC | TAC | AAC | CAC | CTC |      |      |
| 35 | 180 | His | Ala | Ala | Gly | Leu | Gly | Val | Ile | Gln | Asp | Val | Val | Tyr | Asn | His | Leu | 713  |      |
|    | 185 | GGC | CCC | AGC | GGG | AAC | TAC | CTG | CCG | CGG | TTC | GGG | CCG | TAC | CTC | AAG | CAG |      |      |
|    | 190 | Gly | Pro | Ser | Gly | Asn | Tyr | Leu | Pro | Arg | Phe | Gly | Pro | Tyr | Leu | Lys | Gln |      |      |
| 40 | 195 | GGC | GAG | GGT | AAC | ACG | TGG | GGC | GAC | TCG | GTG | AAC | CTG | GAC | GGG | CCC | GGC | 761  |      |
|    | 200 | Gly | Glu | Gly | Asn | Thr | Trp | Gly | Asp | Ser | Val | Asn | Leu | Asp | Gly | Pro | Gly |      |      |
|    | 205 | 225 | TCC | GAC | CAT | GTG | CGC | CGG | TAC | ATC | CTG | GAC | AAC | CTG | GCC | ATG | TGG |      | CTG  |
| 45 | 210 | Ser | Asp | His | Val | Arg | Arg | Tyr | Ile | Leu | Asp | Asn | Leu | Ala | Met | Trp | Leu | 809  |      |
|    | 215 | CGT | GAC | TAC | CGG | GTG | GAC | GGC | CTG | CGG | CTG | GAC | GCC | GTC | CAC | GCC | CTG |      |      |
|    | 220 | Arg | Asp | Tyr | Arg | Val | Asp | Gly | Leu | Arg | Leu | Asp | Ala | Val | His | Ala | Leu |      |      |
| 50 | 225 | AAG | GAT | GAG | CGG | GCG | GTG | CAC | ATC | CTG | GAG | GAC | TTC | GGG | GCG | CTG | GCC | 905  |      |
|    | 230 | Lys | Asp | Glu | Arg | Ala | Val | His | Ile | Leu | Glu | Asp | Phe | Gly | Ala | Leu | Ala |      |      |
|    | 235 | 275 | GAT | CAG | ATC | TCC | GCC | GAG | GTG | GGA | CGG | CCG | CTG | ACG | CTC | ATC | GCC |      | GAG  |
| 55 | 240 | Asp | Gln | Ile | Ser | Ala | Glu | Val | Gly | Arg | Pro | Leu | Thr | Leu | Ile | Ala | Glu | 1001 |      |
|    | 245 | 290 | TCC | GAC | CTC | AAC | AAC | CCG | CGG | CTG | CTG | TAC | CCG | CGG | GAC | GTC | AAC |      | GGG  |
|    | 250 | 305 | Ser | Asp | Leu | Asn | Asn | Pro | Arg | Leu | Leu | Tyr | Pro | Arg | Asp | Val | Asn |      | Gly  |
| 60 | 255 | TAC | GGG | CTG | GAA | GGG | CAG | TGG | AGC | GAC | GAC | TTC | CAC | CAC | GCC | GTC | CAC | 1049 |      |
|    | 260 | Tyr | Gly | Leu | Glu | Gly | Gln | Trp | Ser | Asp | Asp | Phe | His | His | Ala | Val | His |      |      |
|    | 265 | 325 | GTC | AAC | GTC | ACC | GGC | GAA | ACC | ACC | GGC | TAC | TAC | AGT | GAC | TTC | GAC |      | TCG  |
| 65 | 270 | Val | Asn | Val | Thr | Gly | Glu | Thr | Thr | Gly | Tyr | Tyr | Ser | Asp | Phe | Asp | Ser | 1145 |      |
|    | 275 | 330 | CTG | GCC | GCC | CTC | GCC | AAG | GTG | CTC | CGG | GAC | GGC | TTC | TTC | CAC | GAC |      | GGC  |
|    | 280 | 335 | Leu | Ala | Ala | Leu | Ala | Lys | Val | Leu | Arg | Asp | Gly | Phe | Phe | His | Asp |      | Gly  |
| 70 | 285 | 340 | AGC | TAC | TCC | AGC | TTC | CGG | GAA | CGC | CAC | CAC | GGA | CGG | CCG | ATT | AAT | TTC  | 1193 |
|    | 290 | 345 | Ser | Tyr | Ser | Ser | Phe | Arg | Arg | His | His | Gly | Arg | Pro | Ile | Asn | Phe |      |      |
|    | 295 | 350 | AGC | GCC | GTA | CAC | CCA | GCC | GCC | CTG | GTG | GTC | TGT | TCG | CAG | AAC | CAC | GAC  |      |
| 75 | 300 | Ser | Ala | Val | His | Pro | Ala | Ala | Leu | Val | Val | Cys | Ser | Gln | Asn | His | Asp | 1241 |      |
|    | 305 | 355 | 360 | 365 | 370 | 375 | 380 |     |     |     |     |     |     |     |     |     |     |      |      |
|    | 310 | 360 | 365 | 370 | 375 | 380 | 385 |     |     |     |     |     |     |     |     |     |     |      |      |

385 390 395 400  
 5 CAG ATC GGC AAC CGT GCC ACG GGG GAC CGG CTC TCC CAG ACC CTG CCG 1337  
 Gln Ile Gly Asn Arg Ala Thr Gly Asp Arg Leu Ser Gln Thr Leu Pro  
 405 410 415  
 TAC GGA AGC CTG GCC CTC GCT GCG GTG CTG ACC CTG ACG GGA CCC TTC 1385  
 Tyr Gly Ser Leu Ala Leu Ala Val Leu Thr Leu Thr Gly Pro Phe  
 420 425 430  
 10 ACG CCC ATG CTG CTC ATG GGC GAG GAG TAC GGC GCC AGC ACG CCG TGG 1433  
 Thr Pro Met Leu Leu Met Gly Glu Glu Tyr Gly Ala Ser Thr Pro Trp  
 435 440 445  
 CAG TTT TTC ACC TCG CAC CCG GAG CCG GAG CTC GGC AAG GCC ACC GCG 1481  
 Gln Phe Thr Ser His Pro Glu Pro Glu Leu Gly Lys Ala Thr Ala  
 450 455 460  
 15 GAG GGC CGG ATC AAG GAG TTC GAG CGC ATG GGG TGG GAT CCC GCC GTC 1529  
 Glu Gly Arg Ile Lys Glu Phe Glu Arg Met Gly Trp Asp Pro Ala Val  
 465 470 475 480  
 GTG CCC GAT CCC CAG GAT CCT GAG ACG TTC CGC CGG TCC AAG CTG GAC 1577  
 Val Pro Asp Pro Gln Asp Pro Glu Thr Phe Arg Arg Ser Lys Leu Asp  
 485 490 495  
 20 TGG GCG GAA GCC GCC GAA GGC GAC CAT GCC CGG CTG CTG GAG CTG TAC 1625  
 Trp Ala Glu Ala Ala Glu Gly Asp His Ala Arg Leu Leu Glu Leu Tyr  
 500 505 510  
 CGT TCG CTC ACC GCC CTG CGC CGC TCC ACG CCG GAC CTC ACC AAG CTG 1673  
 Arg Ser Leu Thr Ala Leu Arg Arg Ser Thr Pro Asp Leu Thr Lys Leu  
 515 520 525  
 25 GGC TTC GAG GAC ACG CAG GTG GCG TTC GAC GAG GAC GCC CGC TGG CTG 1721  
 Gly Phe Glu Asp Thr Gln Val Ala Phe Asp Glu Asp Ala Arg Trp Leu  
 530 535 540  
 CGG TTC CGC CGG GGT GGC GTG CAG GTG CTG CTC AAC TTC TCG GAA CAG 1769  
 Arg Phe Arg Arg Gly Gly Val Gln Val Leu Leu Asn Phe Ser Glu Gln  
 545 550 555 560  
 CCC GTG AGC CTG GAC GGG GCG GGC ACG GCC CTG CTG GCC ACC GAC 1817  
 Pro Val Ser Leu Asp Gly Ala Gly Thr Ala Leu Leu Leu Ala Thr Asp  
 565 570 575  
 GAC GCC GTC CGG CTA GAA GGT GAG CGT GCG GAA CTC GGT CCG CTG AGC 1865  
 Asp Ala Val Arg Leu Glu Gly Glu Arg Ala Glu Leu Gly Pro Leu Ser  
 580 585 590  
 GCC GCC GTC GTC AGC GAC 1883  
 Ala Ala Val Val Ser Asp  
 595  
 TGACGTTTTC TTGGGGGCGG CGTCCACCGC CGGTGACCGG ATGGTGGACG TCCGCCCGGA 1943  
 AGCCTCGGCG CGGCTGGCAG GATGGAACGC ATGACTTATG TGGCCTCGGA CACCCGCTAC 2003  
 40 GACACCATGC CCTACGCGC CGTCGGACGC AGCGGCCTCA AACTGCCGGC CAT 2056

(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH:6  
 (B) TYPE:amino acid  
 (D) TOPOLOGY:linear  
 (ii) MOLECULE TYPE:peptide  
 (xi) SEQUENCE DESCRIPTION:SEQ ID NO:13:

50 Phe Asp Ile Trp Ala Pro  
 5

(15) INFORMATION FOR SEQ ID NO:14:

55

5 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:17 base pairs  
 (B)TYPE:nucleic acid  
 (D)TOPOLOGY:linear  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:14:

10 TTYGAYATHT GGGCNCC

17

(16) INFORMATION FOR SEQ ID NO:15:  
 (i)SEQUENCE CHARACTERISTICS:  
 15 (A)LENGTH:5  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:15:

20 Asp Trp Ala Glu Ala  
 5

25 (17) INFORMATION FOR SEQ ID NO:16:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:17 base pairs  
 (B)TYPE:nucleic acid  
 (D)TOPOLOGY:linear  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:16:

30

GTAAAACGAC GGCCAGT

17

35 (18) INFORMATION FOR SEQ ID NO:17:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:17 base pairs  
 (B)TYPE:nucleic acid  
 (D)TOPOLOGY:linear  
 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:17:

40

ATGGGNTGGG AYCCNGC

17

45 (19) INFORMATION FOR SEQ ID NO:18:  
 (i)SEQUENCE CHARACTERISTICS:  
 (A)LENGTH:6  
 (B)TYPE:amino acid  
 (D)TOPOLOGY:linear  
 (ii)MOLECULE TYPE:peptide  
 50 (xi)SEQUENCE DESCRIPTION:SEQ ID NO:18:

Met Gly Trp Asp Pro Ala  
 5

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(20) INFORMATION FOR SEQ ID NO:19:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH:14 base pairs  
     (B) TYPE:nucleic acid  
     (D) TOPOLOGY:linear  
 (xi) SEQUENCE DESCRIPTION:SEQ ID NO:19:

TAYGAYGTNT GGGC

14

(21) INFORMATION FOR SEQ ID NO:20:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH:5  
     (B) TYPE:amino acid  
     (D) TOPOLOGY:linear  
 (ii) MOLECULE TYPE:peptide  
 (xi) SEQUENCE DESCRIPTION:SEQ ID NO:20:

Try Asp Val Trp Ala  
 5

## Claims

1. A DNA encoding an enzyme which releases trehalose from a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.
2. The DNA as claimed in claim 1, wherein said enzyme has the following physicochemical properties of:
  - (1) Molecular weight  
 About 57,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); and
  - (2) Isoelectric point (pI)  
 About 3.3-4.6 on isoelectrophoresis.
3. The DNA as claimed in claim 1, wherein said enzyme has an amino acid sequence selected from the group consisting of those as shown in the attached SEQ ID NOs:2 and 4 that initiate from the N-terminal, and homologous amino acid sequences to these amino acid sequences.
4. The DNA as claimed in claim 1, which has a base sequence selected from the group consisting of those as shown in the attached SEQ ID Nos:1 and 3 that initiate from the 5'-terminus, homologous base sequences to the base sequences, and complementary base sequences to these base sequences.
5. The DNA as claimed in claim 4, wherein one or more bases in SEQ ID NOs:1 and 3 are replaced with other bases by means of degeneracy of genetic code without alternating their corresponding amino acid sequences as shown in the attached SEQ ID NOs: 2 and 4.
6. The DNA as claimed in claim 1, which has a base sequence selected from the group consisting of those as shown in the attached SEQ ID NOs:11 and 12.
7. The DNA as claimed in any one of the preceding claims, which is derived from a microorganism selected from the group consisting of those of the genera *Rhizobium*, *Arthrobacter*, *Brevibacterium* and *Micrococcus*.
8. A replicable recombinant DNA containing the DNA as claimed in any one of the preceding claims, and a self-replicable vector.

9. The replicable recombinant DNA as claimed in claim 8, wherein said self-replicable vector is a plasmid vector Bluescript II SK(+).
- 5 10. A transformant obtainable by introducing into a host a replicable recombinant DNA which contains a self-replicable vector and the DNA as claimed in any one of claims 1 to 7.
11. The transformant as claimed in claim 10, which forms an enzyme that releases trehalose from a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.
- 10 12. The transformant as claimed in claim 11, wherein said enzyme has the following physicochemical properties of:
  - (1) Molecular weight  
About 57,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); and
  - 15 (2) Isoelectric point (pI)  
About 3.3-4.6 on isoelectrophoresis.
13. The transformant as claimed in claim 11, wherein said enzyme has an amino acid sequence selected from the group consisting of those as shown in the attached SEQ ID NOs:2 and 4 that initiate from the N-terminal, and homologous amino acid sequences to these amino acid sequences.
- 20 14. The transformant as claimed in any one of claims 10 to 13, wherein said host is a microorganism of the species *Escherichia coli*.
- 25 15. A recombinant enzyme which releases trehalose from a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.
- 30 16. The recombinant enzyme as claimed in claim 15, which has the following physicochemical properties of:
  - (1) Molecular weight  
About 57,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); and
  - (2) Isoelectric point (pI)  
About 3.3-4.6 on isoelectrophoresis.
- 35 17. The recombinant enzyme as claimed in claim 15, which has an amino acid sequence selected from the group consisting of those as shown in the attached SEQ ID NOs:2 and 4 that initiate from the N-terminal, and homologous amino acid sequences to these amino acid sequences.
- 40 18. The recombinant enzyme as claimed in claim 15, which is encoded by a DNA according to any one of claims 4 to 6.
19. A process for producing a recombinant enzyme, which comprises culturing the transformant as claimed in claim 10 to form a recombinant enzyme according to any one of claims 15 to 18.
- 45 20. The process as claimed in claim 19, wherein the transformant is inoculated into a liquid culture medium having a pH of 2-8, and cultured at a temperature of 25-65°C for about 1-6 days.
- 50 21. The process as claimed in claim 19 or claim 20, wherein the collecting step of the recombinant enzyme is effected by one or more methods selected from the group consisting of centrifugation, filtration, concentration, salting out, dialysis, ion-exchange chromatography, gel filtration chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectrophoresis.
- 55 22. A method for converting a non-reducing saccharide, which contains a step of allowing the recombinant enzyme as claimed in any one of claims 15 to 18 to act on a non-reducing saccharide, having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher and, to release trehalose.

23. The method as claimed in claim 22, wherein said non-reducing saccharide is prepared by successively treating a member selected from the group consisting of starch, amylopectin, amylose and mixtures thereof with acid together with or without amylase, and subjecting the resultant mixture to the action of a non-reducing saccharide-forming enzyme.

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24. The method as claimed in claim 22, wherein said non-reducing saccharide is a member selected from the group consisting of  $\alpha$ -glucosyltrehalose,  $\alpha$ -maltoosyltrehalose,  $\alpha$ -maltotriosyltrehalose,  $\alpha$ -maltotetraosyltrehalose,  $\alpha$ -maltopentaosyltrehalose, and mixtures thereof.

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25. The method as claimed in any one of claims 22 to 24, wherein said non-reducing saccharide is in a solution form with a concentration of 50 w/v% or lower, and the step is carried out at a temperature of about 40-55°C and a pH of about 6-8.

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FIG. 1

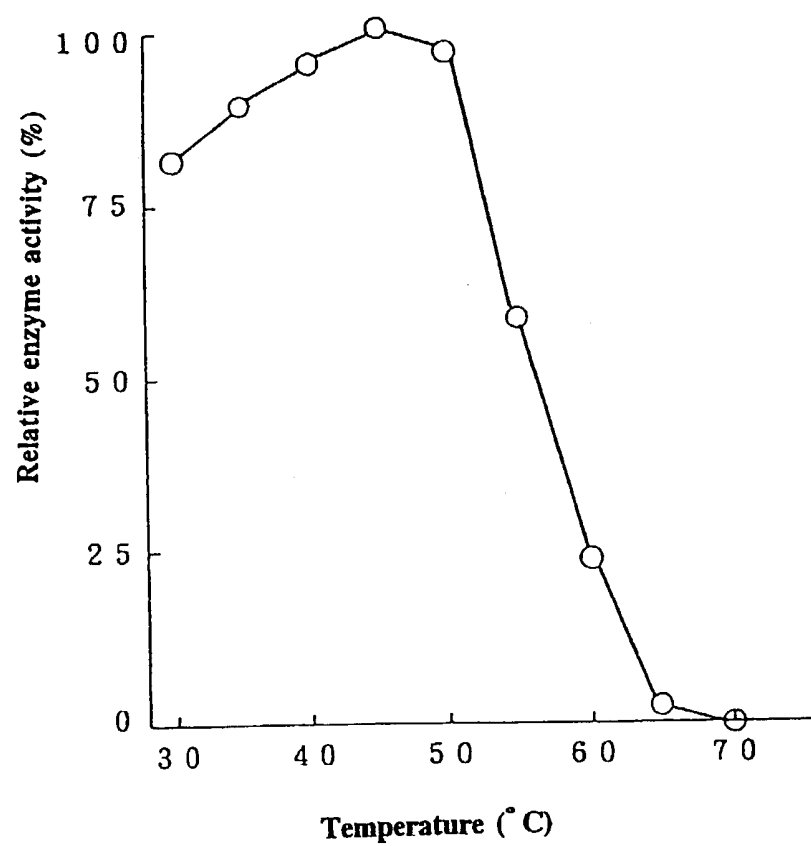


FIG. 2

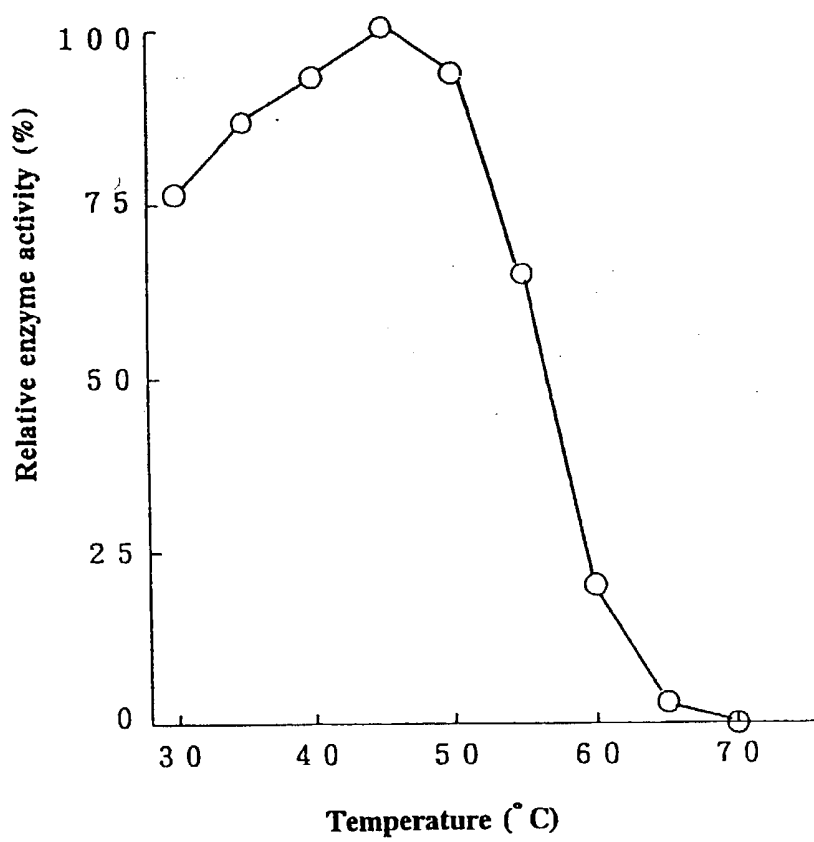


FIG. 3

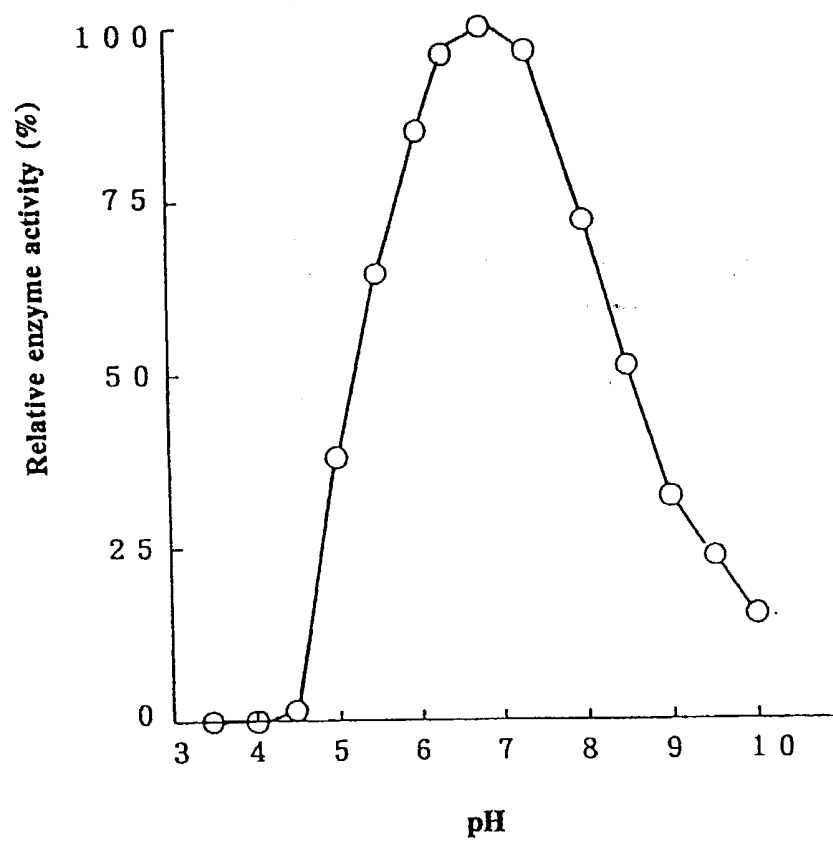


FIG. 4

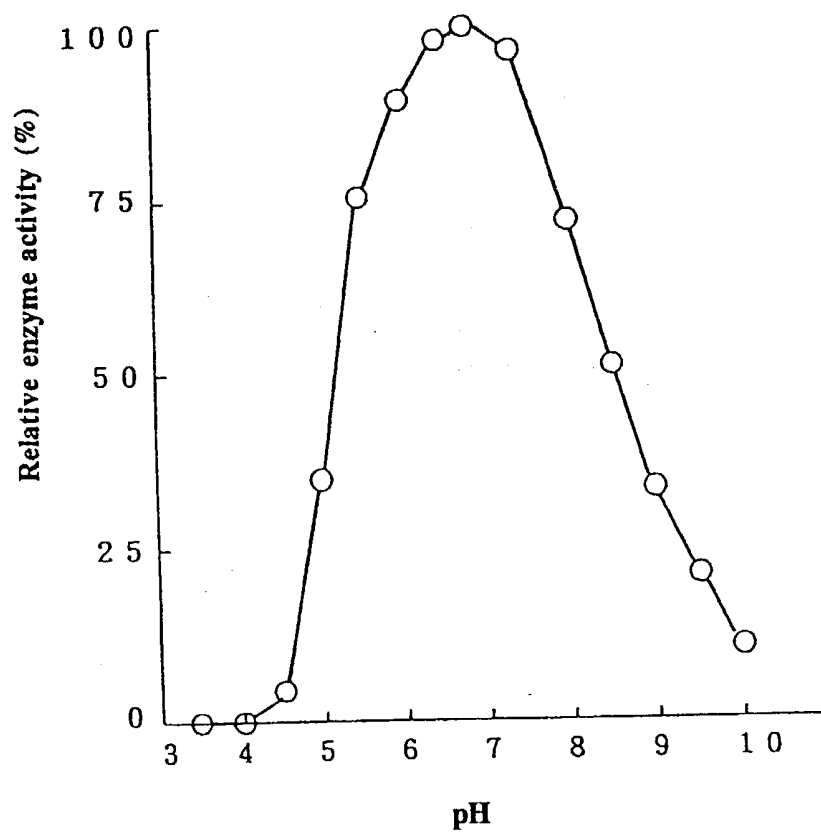


FIG. 5

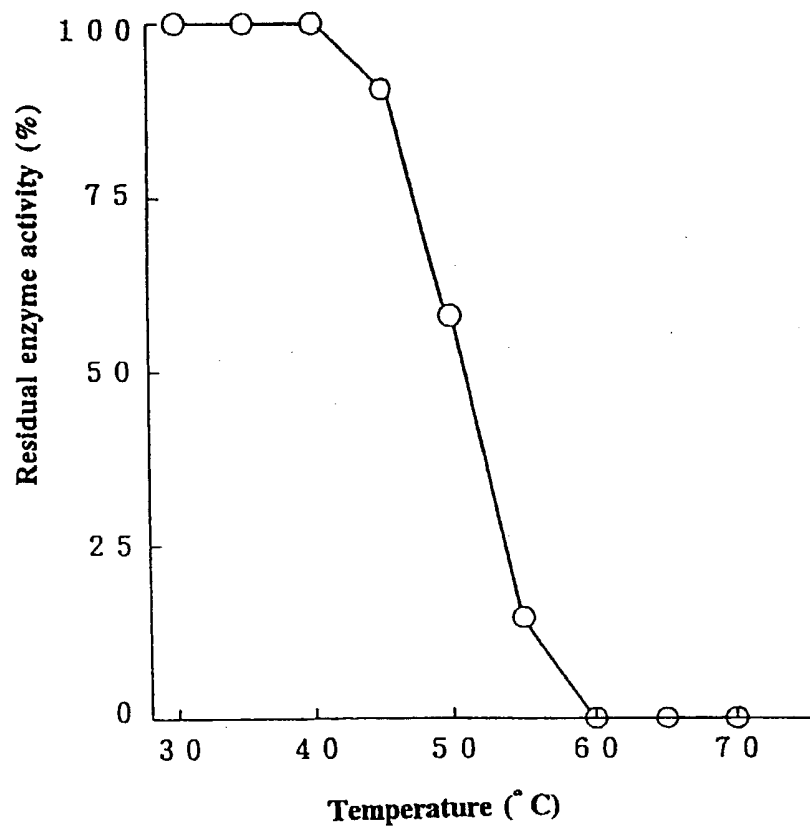




FIG. 6

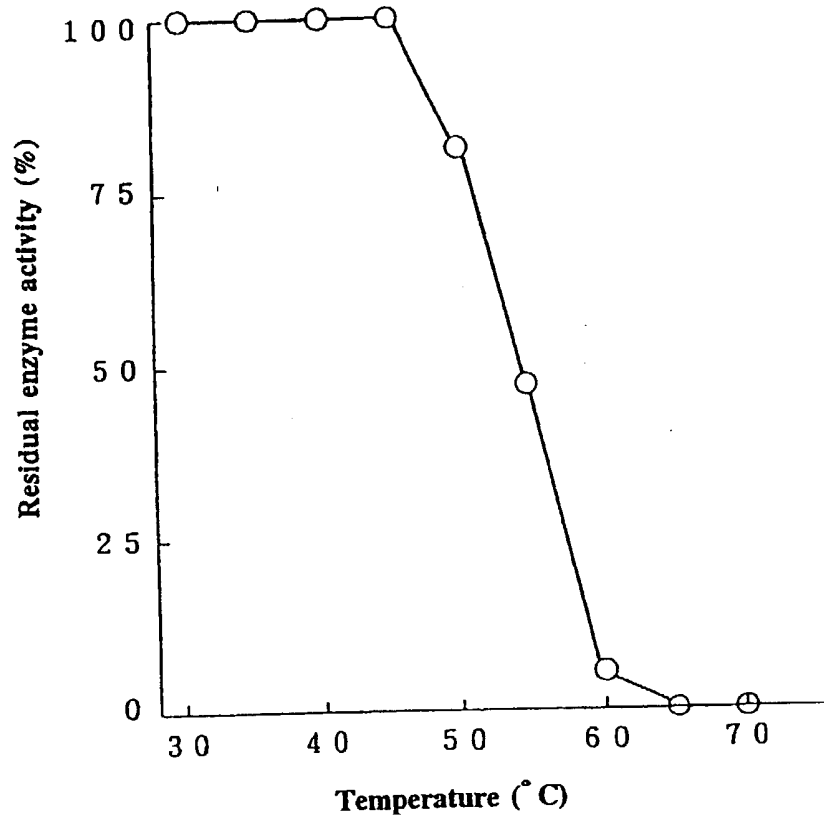


FIG. 7

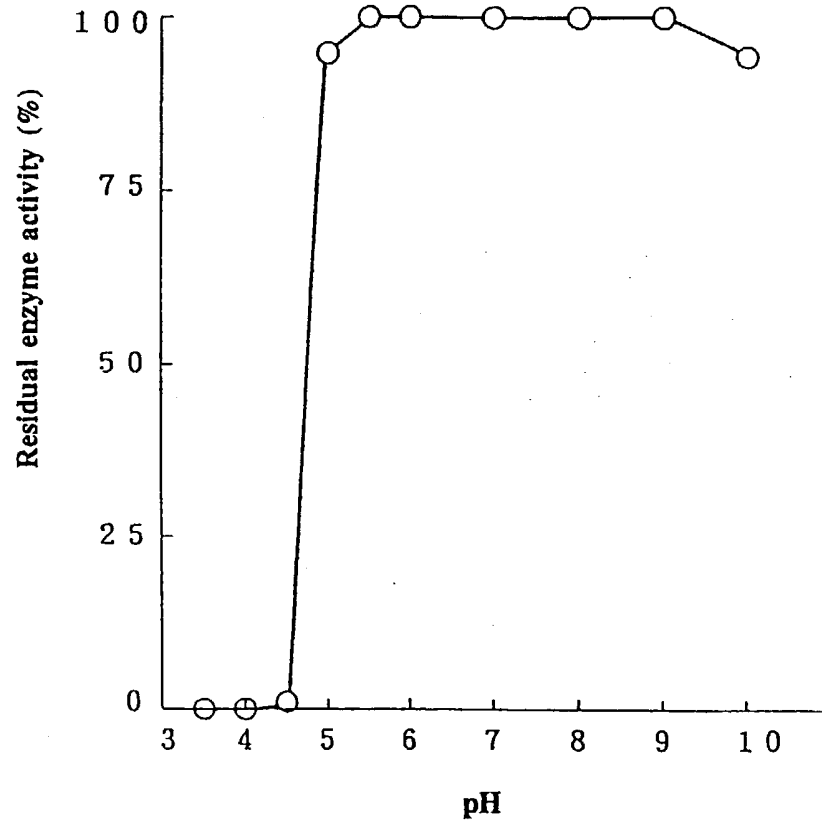


FIG. 8

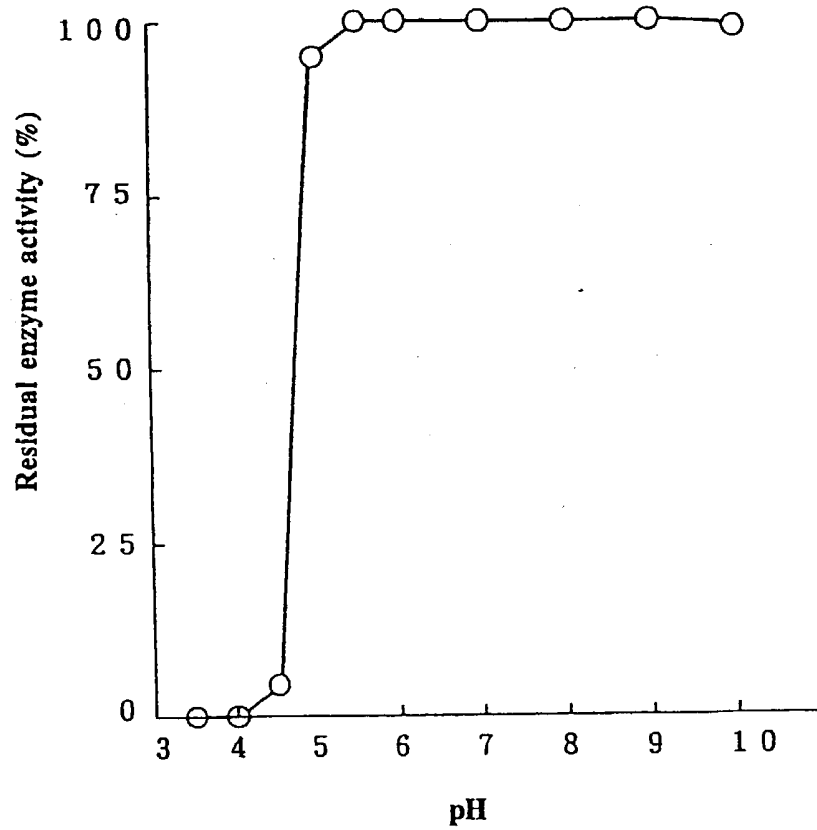


FIG. 9

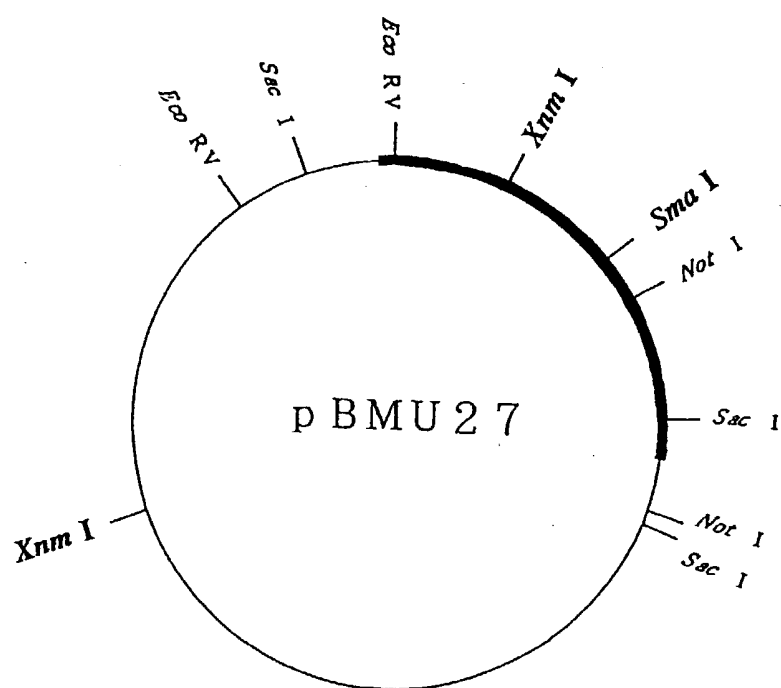


FIG. 10

